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Working Group "Integrated Control in Oilseed Crops"

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Groupe de Travail "Lutte Intégrée en Culture d'Oléagineux"

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Dedication

Bent Bromand in memoriam

Bent Bromand, the first Convenor of the IOBC Working Group on Oilseed Rape sadly died last year. Bent was Danish and worked for most of his life as an entomologist and field trial officer at the Danish Research Centre for Plant Protection.

I got to know him in a Scandinavian project on the pollen beetle in the mid 70's. Around 1980 Bent talked with me about setting up a Working Group on oilseed rape within IOBC, but the IOBC Council wanted us to investigate the possibility of forming a subgroup under the IOBC group ''Integrated control in Brassicas" at that time led by Dr. Tom Coaker, U.K. This group had a focus on the cabbage root fly. Bent and I went to Gent in Belgium where we met with the Brassica group on the 12 June 1981. Dr Coaker reported to the Council that the differences between the existing Brassica group and a possible remit for an oilseed rape group were too great and recommended that a separate Working Group on ''Integrated control in oilseed rape" be set up. This was accepted.

Bent became the first Convenor of the new Working Group and the first meeting was held in Copenhagen on the 21 and 22 March 1982. Most of the founder members are now retired – Wilhelm Krüger, Christer Svensson, Howard Gould and Jacques Lerin just to mention 2 pathologists and 2 entomologists.

Bent grew up on a farm in south Jutland and all his life he kept the farmers perspective in his research. He was always optimistic and with a glint in his eye. Nothing seemed to disturb his inner balance – and I was not surprised when he told me that he had started as a Phantom-fighter pilot, flying in Canada. This was not really what he had intended and soon he started his MSc studies in agronomy. Last year in May he retired to pay more attention to family, garden and hobbies – but died just 2 month later on the 17 July 2003.

I suggest that we dedicate this Working Group meeting in the honour of this very nice person and the creator and first convenor of this Working Group.

Christer Nilsson, 30 March 2004

Preface

We dedicate this issue to Bent Bromand, who was the first convenor of our working group founded by him and some colleagues in 1982. Bent died on 17 July 2003, just a short while after he retired. On behalf of IOBC and all group members, we gratefully acknowledge his engagement in leading the working group "Integrated Control in Oilseed Crops" until 1990. We will keep him in honourable reminiscence. Christer Nilson kindly wrote a paragraph to let the younger people know about him and his work.

After our last meeting in 2002 in Soest, Germany, it was decided to meet this year at Rothamsted Research. Our Rothamsted colleagues took over the responsibility in organising the meeting and did a great job. Thanks to all of you involved in organising this meeting! IOBC is also grateful to RRes for hosting the meeting without charge, save expenses involved with printing the abstract booklets and provision of refreshments. IOBC really appreciates such support very much.

We also have to report here some changes since the time of the last and our most recent meetings. Ingrid Williams (Rothamsted Research, U.K.) and Volker Paul (FH Soest, Germany) lay down their convenorship. The two were the head of our group during the time period of 1990 to 2003/04. Volker Paul communicated his decision during a small IOBC group meeting beside the 11th International Rapeseed Conference held in Copenhagen in 2003. Birger Koopmann (University of Göttingen, Germany) was elected to follow up Volker Paul and accepted. During the biannual meeting at Rothamsted, Ingrid Williams also resigned her convenorship. Sam Cook (Rothamsted Research, U.K.) was selected to take over the responsibility for the entomology group. We both want to express our sincere gratitude on behalf of IOBC and all group members to Ingrid and Volker. Thanks a lot for everything you did in the past for IOBC in general and especially for our group. After our first meeting and the preparation of the Bulletin, we know what a bunch of work this was! We hope that both of you will support us in the future as we try to fulfil the job to a similar high standard as both of you achieved before. You will be a hard act to follow and we all owe the current success of this group to you both.

We gratefully acknowledge all authors who provided a paper for the bulletin and who considered the formatting. This helped a great deal in keeping our work load to a low level. We also express our gratitude to Ingrid Williams and Neal Evans for their reviewing and editing work, which was of high value to all of us. We hope that authors will find their contributions presented here in a proper manner and that readers will find information of value to their own work.

Birger Koopmann and *Sam Cook*

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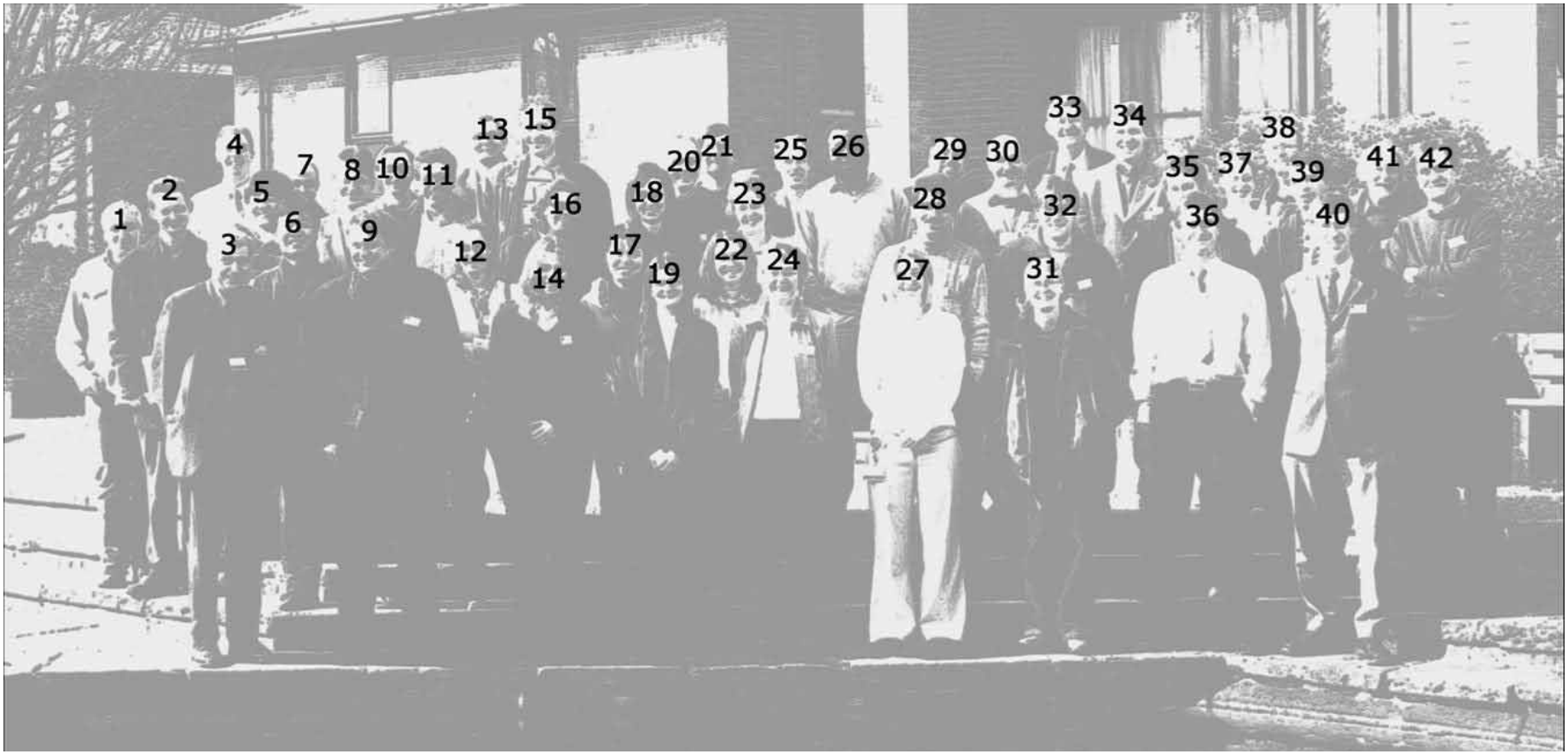

1 Piotr Kacklicki; 2 Dave Frearson; 3 Bruce D.L. Fitt; 4 Neal Evans; 5 Ze (Arthur)Liu; 6 Ziqin Le; 7 Xavier Pinochet; 8 Daniel Nerad; 9 Oliver Schlein; 10 Zbigniew Karolewski; 11 David Kenyon; 12 Marie-Hélène Balesdent; 13 Zdislaw Klukowski; 14 Sarah Dunker; 15 Witold Irzykowski; 16 Anna Stachowiak; 17 Yong-Ju Huang; 18 Julia Olechnowicz; 19 Samantha Cook; 20 Andrew Ferguson; 21 Heikki Hokkanen; 22 Malgorzata Jedrycska; 23 Jane Thomas; 24 Ingrid Williams; 25 Peter Gladders; 26 Akinwunmi (Olu)Latunde-Dada; 27 Emma Smith; 28 Hassan Barari; 29 Wolfgang Büchs; 30 Nash Nashaat; 31 Ingrid Happstadius; 32 Matt Skellern; 33 Christer Nilsson; 34 Holger Kreye; 35 Nigel Watts; 36 Ross Piper; 37 Elizabeth Pirie; 38 Martin Jonsson; 39 João Carlos da Silva Dias; 40 Jonathan West; 41 Alastair McCartney; 42 Bernd Ulber

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The EU project MASTER (MAnagement STrategies for European Rape pests): a review of progress

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Abstract: The EU-funded project 'Integrated pest management strategies incorporating bio-control for European oilseed rape pests' (QLK5-CT-2001-01447) (acronymn MASTER for MAnagement STrategies for European Rape pests) is targeting the six most important European Rape Pests: *Psylliodes chrysocephala* (cabbage stem flea beetle), *Meligethes aeneus* (pollen beetle), *Ceuto-rhynchus assimilis* (cabbage seed weevil), *Ceutorhynchus napi* (rape stem weevil), *Ceutorhynchus pallidactylus* (cabbage stem weevil) and *Dasineura brassicae* (brassica pod midge). This paper reports progress on the project's five scientific objectives: 1. To determine the identity, status and potential of bio-control agents for rape pests in Europe, to increase knowledge of their ecology and identify key factors affecting their efficacy, 2. To develop economically-viable, environmentally-acceptable IPM strategies for European rape that maximise bio-control of target pests and minimise pesticide use, 3. To determine the socio-economic feasibility, importance and economic efficiency of the IPM strategies in Europe and to assess the socio-economic factors influencing their adoption, 4. To construct a Phenological Model of target pests and their bio-control agents, relating occurrence on the crop to growth stage and climatic/weather conditions, for integration into existing Decision Support systems, 5. To produce Technical Guidelines on the IPM strategies for end-users. Information about key natural enemies has been collated and published. Further information can be found on the project website www.iacr.bbsrc.ac.uk/pie/master/master.htm

Key words: insect pests - biocontrol - decision support - oilseed rape - IPM strategies

Introduction

MASTER (MAnagement STrategies for European Rape pests) is the acronym for an EU-funded project, entitled 'Integrated pest management strategies incorporating bio-control for European oilseed rape pests' (QLK5-CT-2001-01447). The project is of four years' duration and was initiated in December 2001. The project consortium has partners from six EU countries, namely, Estonia, Finland, Germany, Poland, Sweden and the UK. The main objective of the project is to construct, develop, evaluate and promote an Integrated Pest Management (IPM) System for the European winter oilseed rape crop incorporating biological control of pests (Williams *et al*., 2002). MASTER has five scientific objectives. This paper reviews progress on each objective in turn over the first two years of the project.

Objective 1: To determine the identity, status and potential of bio-control agents for rape pests in Europe, to increase knowledge of their ecology (biology, phenology, behaviour and habitat requirements) so as to identify key factors affecting their efficacy.

The six pests of winter oilseed rape in Europe to be targetted by the project have been agreed (Table 1) viz. *Psylliodes chrysocephala* (cabbage stem flea beetle), *Meligethes aeneus* (pollen beetle), *Ceutorhynchus assimilis* (cabbage seed weevil), *Ceutorhynchus napi* (rape stem weevil), *Ceutorhynchus pallidactylus* (cabbage stem weevil) and *Dasineura brassicae* (brassica pod midge). These attack the crop successively at various stages of its growth and damage different parts of the plant (Alford *et al.*, 2003).

Literature on the naturally-occurring parasitoids, predators and pathogens that attack the six target pests has been reviewed and collated as a book, the first of its kind on this subject (Alford, 2003). The most important species for bio-control have been identified and are the focus of the project (see Tables 1 & 2).

Gaps in our knowledge of the natural enemies are being addressed through strategic research in three Workpackages: Parasitoids (led by Bernd Ulber), Predators (led by Wolfgang Büchs) and Pathogens (led by Heikki Hokkanen). This research investigates aspects of their biology, abundance, phenology, distribution, feeding preferences and host location important to underpin the development of IPM strategies for the crop.

Pests and parasitoids

Published literature on the parasitoids attacking the six target pests has been reviewed by partners (for *P. chrysocephala* see Ulber & Williams, 2003; for *C. napi* and *C. pallidactylus* see Ulber, 2003; for *M. aeneus* see Nilsson, 2003; for *C. assimilis* see Williams, 2003a; for *D. brassicae* see Williams, 2003b). In total, the six target pests are host to *ca.* 88 species of parasitoid; most are hymenopterous wasps, especially braconids, chalcids and ichneumonids, and most attack the larval stages of the host. At least 11 species are considered to be sufficiently widespread and abundant in partner countries to be of potential economic importance for biocontrol and these are now the key parasitoid species for the project (Table 1). Taxonomy and correct identification of these species is proving to be challenging as existing keys are inadequate. All partners are making national collections of key species. New methods for the rearing of parasitoids are being developed (eg. Williams *et al*., 2003; Barari *et al*., 2004).

The effects of plant density, spatial distribution of plants and plant morphology on larval parasitism of stem-boring pests are being studied in Germany. This information will be used in IPM Strategies to manipulate crop canopy structure and parasitoid behaviour to enhance parasitoid efficacy.

In 2001/2 and 2002/3, field experiments investigated the effect of different sowing rates (30 seeds/m^2, 60 seeds/m^2 and 90 seeds/ m^2) and row spacings (13 cm and 40 cm). Plots were sown with a hybrid winter oilseed rape variety, 'ARTUS'; each treatment comprised six replicated plots. Plant establishment, length and diameter of stems, number of leaves and number of side racemes were recorded in the plots in December, April and May.

Table 1. Key larval parasitoids of the MASTER target pests of winter oilseed rape in partner countries

Target pest	Key parasitoid	Family	Occurrence
Psylliodes chrysocephala	*Tersilochus microgaster* (Szépligeti)	Ichneumonidae	UK;P;DE
Ceutorhynchus Napi	*Tersilochus fulvipes* (Gravenhorst)	Ichneumonidae	P;DE
Ceutorhynchus pallidactylus	*Tersilochus obscurator* Aubert	Ichneumonidae	UK;P;DE
Meligethes aeneus	*Phradis interstitialis* (Thomson)	Ichneumonidae	UK;SE;P;DE
	Phradis morionellus (Holmgren)	Ichneumonidae	UK;SE;EE;P;DE
	Tersilochus heterocerus Thomson	Ichneumonidae	UK;SE;P;DE
Ceutorhynchus assimilis	*Trichomalus perfectus* (Walker)	Pteromalidae	UK;SE;EE;P;DE
	Stenomalina gracilis (Walker)	Pteromalidae	UK;EE;P;DE
	Mesopolobus morys (Walker)	Pteromalidae	UK;SE;EE;P;DE
Dasineura brassicae	*Platygaster subuliformis* (Kieffer)	Platygastridae	UK;SE;EE;P;DE
	Omphale clypealis (Thomson)	Eulophidae	UK;SE;EE;DE

To assess the abundance and within-plant distribution of stem-boring larvae of pests, plant samples were collected in March (*P. chrysocephala*), April and May (*P. chrysocephala*, *C. napi*, *C. pallidactylus*). Stems and leaves were dissected and the number of larvae recorded. The level of parasitism of the larvae feeding at various positions within stems and leaves was determined by dissection; sub-samples of full-grown host larvae were reared to adults.

In the plant samples collected in 2001/2, the mean length and diameter of stems, the mean number of lateral racemes and leaves as well as the mean length and diameter of lateral racemes were affected significantly by sowing rates. Plant morphology was little affected in 2002/3. Plant infestation by stem-boring larvae increased as the number of plants/m^2 decreased (Nuss & Ulber, 2004). No clear relationship was found between the parasitism of *C. napi* and *C. pallidactylus* by *T. fulvipes* and *T. obscurator*, respectively, and plant density.

Host larvae feeding in lateral racemes showed a greater percentage of parasitism than host larvae in main stems. At wider row spacing (40cm) the larval parasitism of *P. chrysocephala*, *C. napi* and *C. pallidactylus* was greater than that at narrow row spacing (13 cm). However, this effect was only observed on some of the sampling dates, indicating that the interactions between plant factors and larval parasitism may be influenced by growth stage, the phenologies of hosts and parasitoids and the spatio-temporal within-plant distribution of host larvae.

The effect of host plant architecture as well as within-plant distribution and instar of host larvae on parasitism of *P. chrysocephala* and *C. pallidactylus* was studied further in another field experiment. Application of nitrogen fertiliser to the crop was used to create plots with different crop canopy structure, crop microclimate, plant size, number of leaves and number of side racemes. These factors could indirectly affect the behaviour and host finding success of larval parasitoids. Winter rape *cv*. ARTUS was sown in replicated plots; part of these received a high level of nitrogen (200 kg/ha) while the other part was not supplied with nitrogen. Samples of leaves were collected from different plots at random and by diameter of petiole subdivided in two classes: < 5mm and > 5mm. Larvae of *P. chrysocephala* and *C. pallidactylus* were sampled from the petioles and dissected in the laboratory. Sub-samples of larvae were reared to pupation and the emerging beetles or parasitoids were identified. The number of larvae per leaf and the level of parasitism were not affected significantly by nitrogen usage. However, while the number of *C. pallidactylus* larvae in large petioles (> 5 mm) was twice as high as in small petioles (< 5 mm), the number of larvae of *P. chrysocephala* was not affected significantly by the diameter of petiole. The level of parasitism of *C. pallidactylus* larvae by *T. obscurator* decreased with increasing diameter of petiole. Similar results have been obtained with parasitism of *P. chrysocephala* by *T. microgaster* (Ulber & Wedemeyer, 2004).

The effect of physical and chemical cues on habitat and host location by pests and their parasitoids is being investigated by the UK partner, to find ways in which responses could be manipulated for pest control. The effect of wind direction and wind speed on immigration flights of pests and key parasitoids to the crop was investigated in 2002/3 using 8 malaise and 8 intercept traps placed 5 m from the perimeter of a circular plot (20 m diameter) of winter rape. A meteorological station on site recorded wind direction, wind speed, precipitation and temperature. Insects were trapped daily during October and on a total of 32 days from mid March to early July. Traps caught all species of target pest and key parasitoids, with the malaise traps being most effective for *P. chrysocephala*, *D. brassicae* and parasitoids and the intercept traps being most effective for the other coleopterous pests. Directionality of catch is being analysed in relation to wind direction and wind speed.

The responses of *M. aeneus* to odour from flowers, anthers and pollen have been tested using a linear track olfactometer (Cook *et al.*, 2002). These have shown that the odour of intact rape flowers attracts both males and females, indicating that this pest may locate the rape crop using floral odours as cues. The attractive odour emanates from all floral parts: petals/sepals, anthers and pollen. Floral samples with anthers were more attactive than those without anthers. The effect of pollen on oviposition, and on survival and development of *M. aeneus* larvae was investigated using Synergy, a composite hybrid oilseed rape variety comprising male-fertile (with pollen) and male-sterile (without pollen) plants (Cook *et al*., 2003). Adult beetles on racemes from male-fertile and male-sterile plants within a crop were counted, collected and their sex determined. Buds were examined for oviposition holes, eggs and larvae. Flowers were examined for first and second instar larvae. Larvae were reared in flowers, with or without pollen under controlled conditions and their weight and survival monitored. On Synergy, female *M. aeneus* were more abundant on male-fertile than male-

sterile plants during flowering. More male-fertile than male-sterile buds were accepted for oviposition. Although numbers of first instar larvae did not differ, more second instars were found on male-fertile than male-sterile flowers, indicating better survival. A diet including pollen increased larval survival and pupal and adult weight.

Ways in which the responses of *M. aeneus* to cues and resources from the inflorescence can be used to manipulate the distribution of *M. aeneus* are being investigated. The effect of inflorescence growth stage on their spatial dynamics has been examined using semi-field arrays of rape plants placed in a fallow field (Frearson *et al*., 2004). One array simulated a crop with all plants in green bud, the most vulnerable stage for injury by this pest. The other array simulated a crop with plants in green bud surrounded by a trap crop border with plants in early flower. The beetles were allowed to infest the plants naturally and their distribution on the plants was monitored daily for 10-12 days. Insect and inflorescence growth stage distributions were analysed and compared using Spatial Analysis by Distance IndicEs (SADIE) (Perry, 1998). Inflorescence growth stage had a marked effect on beetle spatial dynamics. The outer trap crop border successfully maintained edge distributions of beetles for at least one week while inner plants were in the vulnerable green bud stage. Females moved to inner plants sooner than males probably in response to the increasing numbers of buds for oviposition.

Predators

The role of predators in winter oilseed rape is less well understood than that of parasitoids but is under study both in the field and using laboratory experiments. While parasitisation rate is a clear and comparatively easy indicator of the importance of a parasitoid species as a natural enemy of a pest, the polyphagous behaviour of most predators makes it more difficult to determine which of the species present are key species for biocontrol within the crop.

Analysis of published literature and other sources shows that more than 160 taxa, mainly belonging to the beetles (Coleoptera: Carabidae and Staphylinidae), the spiders (Arachnida: Araneae), the flies (Diptera: Hybotidae, Dolichopodidae, Muscidae), and the bugs (Heteroptera: Nabidae and Anthocoridae) are reported as dominant and subdominant in fields of oilseed rape (Büchs, 2003); many of these probably attack the target pests. Among them, species of carabid beetle (*Amara similata, Anchomenus dorsalis, Harpalus affinis, Nebria brevicollis, Poecilus cupreus, Pseudoophonus rufipes, Pterostichus melanarius, Trechus quadristriatus*), rove beetle (*Amischa analis, Anotylus rugosus, Atheta spp., Lathrobium fulvipenne, Omalium caesum, O. rivulare, Philonthus cognatus, Tachyporus hypnorum, T. nitidulus*), spider (*Bathyphantes gracilis, Erigone atra, E. dentipalpis, Lepthyphantes tenuis, Meioneta rurestris, Oedothorax apicatus*), Hybotidae (*Platypalpus pallidicornis, P. apllidiventris, P. interstinctus, P. articulatoides*), Dolichopodidae (*Dolichopus acuticornis, Medetera micacea*) and Syrphidae (*Episyrphus balteatus*) have been identified as key predators in winter rape crops. Furthermore, species of other carnivorous taxa like Acarina, Cantharidae (e.g. *Cantharis lateralis*), Coccinellidae (e.g. *Coccinella septempunctata, Propylea quattuordecimpunctata*), Chilopoda (e.g. *Geophilus electricus*), Heteroptera (e.g. *Nabis pseudoferus, Anthocoris* spp.), Histeridae (e.g. *Gramnostethus marginatus*), Malachidae, Neuroptera (e.g. *Chrysoperla carnea*), Opiliones (e.g. *Laccinius ephipiatus, Platybunus trinagularis*), Silphidae (e.g. *Silpha* spp.) and Thysanoptera (e.g. *Aeolothrips* spp.) are likely to be abundant in oilseed rape. Further information about the predator fauna of winter rape is being obtained throughout the course of this project by pitfall trapping in partner countries (see later section under Objective 2).

The feeding preferences and capacities of some predator species are being determined through gut dissections of field-collected specimens, molecular techniques and choice tests in

the laboratory. In the UK, gut dissections of field-collected carabids have revealed that the larvae of *M. aeneus* were eaten in the field by three species of carabid, of which the most abundant in the UK was *N. brevicollis*; there was also some evidence of predation on *Ceutorhynchus* sp. larvae and adults, although this was rare (Piper & Williams, 2004). In Germany, *P. cupreus* and *A. dorsalis* are suspected of highest predation rates on *M. aeneus* larvae. The majority of Carabidae and all the Staphylinidae contained only liquid in their crops and Araneae are obligate liquid feeders; therefore a technique other than gut dissections is needed to further define the diet of these species in the field. Polymerase Chain Reaction (PCR) has been chosen as the diagnostic method. This has been already performed successfully by using specific primers to identify the DNA of *M. aeneus* larvae inside the predators' digestive systems of four key carabid species at different time intervals after prey consumption.

Feeding tests in the laboratory have revealed distinct differences in the degree of carnivory between the three key species of carabid: *A. similata*, *P. rufipes* and *P. cupreus* (Schlein & Büchs, 2004). In choice tests, oilseed rape seeds were offered together with larvae of *D. brassicae*. Surprisingly, *A. similata,* usually considered to be a seed eater (Jorgensen & Toft, 1997), showed a significantly greater degree of entomophagy than *P. rufipes*, whereas no significant difference in the mean consumption rate of pest larvae could be found when compared to *P. cupreus.* The importance of *A. similata,* a species predominant in Central European rape fields (Büchs, 2003; Schlein & Büchs, 2004), thus appears to have been underestimated. This finding of a likely substantial, but to the present date mostly neglected potential predator of larvae of target pests, was confirmed by other non-choice and choice feeding trials.

Choice tests have also shown a significant preference of the common Staphylinid *T. hypnorum* for larvae of *M. aeneus* when this prey was offered simultaneously with larvae of *D. brassicae,* or with the larvae of insects (*Nasonia vitripennis*, Hymenoptera: Pteromalidae) that are alien to the oilseed rape field. Adults of this Staphylinid are able to climb the plants and hunt their prey in the flower heads; *T. hypnorum* is therefore believed to be an influential key predator on *M. aeneus*. Staphylinid larvae are frequently observed together with *M. aeneus* larvae in the flowering canopy of oilseed rape and it seems reasonable to suspect that they also feed on the pest larvae in the field. The numbers of dropping larvae per m² have been recorded by funnel traps which have been installed on the soil under the oilseed rape canopy. The numbers of *M. aeneus* larvae were similar, but in the ICM system, the numbers of Staphylinidae larvae were ten times greater than in the STN system. A temporal coincidence between pests and predators has been detected in the ICM system but not in the STN system.

To determine the role of web spiders as predators and their management related density, the webs of *Theridion impressum* (Araneidae: Theriidae) were counted in winter rape crops during periods of crop colonisation by pests and during the hatching of their new generations in both management systems (STN and ICM). To assess the densities of horizontal webs of Linyphiid spiders pieces of wire mesh were installed in the field and the numbers of mesh units covered with Linyphiid webs were counted in both systems. In both experiments no significant differences between the ICM and the STN system were detected.

Pathogens

Pathogenic organisms exerting natural control of oilseed rape pests include entomopathogenic fungi (epf), nematodes (epn), bacteria and protozoa (Hokkanen *et al*., 2003). To assess the incidence of soil entomopathogenic nematodes and fungi, in 2002/3, soil samples were collected and analysed from 10 oilseed rape fields in each partner country. This survey

showed that the incidence of epf and epn in most soils was too low to provide any appreciable mortality on the target pests. Inundative introduction of these biocontrol agents may be feasible, possibly followed by measures to conserve them in the soil environment after application. Two pathogenic organisms, the epn *Steinernema feltiae* and the epf *Metarhizium anisopliae* have been selected for this approach.

In Finland, application of *S. feltiae* to the soil, at the rate of 1 million infective juveniles/m^2, shortly before the pupation of *M. aeneus*, decreased adult emergence by 94%, but application two months earlier (at the time of sowing of spring rape) did not significantly lower pest numbers (Menzler-Hokkanen & Hokkanen, 2004). An inoculation and conservation strategy using the epn *Steinernema feltiae* will be tested in the coming year as part of the IPM strategy (Objective 2) in the different partner countries.

Previous trials in Finland have shown that the epf *M. anisopliae*, when applied into the soil at the time of sowing or as a spray at the time of flowering, will significantly reduce *M. aeneus* and *Phyllotreta* spp. numbers (Hokkanen *et al*., unpublished). Earlier studies in the UK, have further shown that this epf can be disseminated by honey bees to the flowering canopy of oilseed rape, where it will infect *M. aeneus* (Butt *et al*., 1998) and further work is now underway to investigate whether this method of delivery can also be used to infect *C. assimilis* in the crop canopy.

Spatio-temporal studies

Within-field synchrony and co-incidence of the pests and their natural enemies are being investigated in the UK, in Germany, and in Poland to give a more detailed and informative picture of crop colonisation than hitherto achieved and to aid precision treatment timing and targetting for natural enemy conservation.

In the UK, the focus has been on the phenology and distributions of *M. aeneus* (Ferguson *et al*., 2003) and *D. brassicae* (Ferguson *et al*., 2004), and their endoparasitoids, and on carabids and pest larvae (Warner, 2001; Warner *et al*., 2003). Insects were sampled at 40 spatially-referenced points within a crop of winter rape and the following crop of winter wheat. Spatial distributions were analysed and compared using Spatial Analysis by Distance Indices (SADIE). In this study, both adult and larval *M. aeneus* distributions showed clustering in the north-eastern half of the field (Ferguson *et al*., 2003); the distribution of *P. interstitialis* larvae was associated with that of their host whereas the eggs of *T. heterocerus* were spread evenly across the field. A quarter of *M. aeneus* larvae were parasitised by *Phradis interstitialis* and *Tersilochus heterocerus* but fewer than two percent of parasitised hosts gave rise to parasitoid adults, indicating heavy overwintering losses. Adult *P. interstitialis* emerged from overwintering two weeks before *T. heterocerus* which was the more abundant.

Spatio-temporal studies in *D. brassicae* (Ferguson *et al.*, 2004), showed that pre-diapause, the start of emergence of both key endoparasitoid species attacking this pest, namely *Omphale clypealis* and *Platygaster subuliformis* was coincident with the emergence of adult *D. brassicae* but that the emergence of parasitoids was more prolonged. Emergence of *O. clypealis* post-diapause peaked a month later than either *D. brassicae* or *P. subuliformis*. All insects were markedly edge-distributed and spatially associated pre-diapause but only *O. clypealis* remained edge-distributed post-diapause. Mortality of life stages spent in the soil is high. Only 7% of first generation larvae gave rise to emerging insects pre-diapause and 0.2% of first and second generation larvae to emerging insects post-diapause. Parasitoids comprised 42% and 49% of insects emerging from *D. brassicae* cocoons pre-diapause and post-diapause, respectively. The endoparasitoid *O. clypealis* was more abundant than the endoparasitoid *P. subuliformis* in 1999.

Pests are most vulnerable to predation by carabids when in the soil, ie at the egg and young larval stages, as in *P. chrysocephala*, or as mature larvae or pupae in the soil, as in *M. aeneus, C. assimilis* and *D. brassicae*. Three carabid species, *T. quadristriatus, P. madidus* and *N. brevicollis* have been found to be active during peak immigration and egg-laying of *P. chrysocephala* into the crop (Warner *et al.*, 2003). The first two species showed significant spatial association with the larvae of *P. chrysocephala* during October. In feeding studies, only *T. quadristriatus* consumed the eggs of *P. chrysocephala* suggesting that this species may be the most important predator of the pest in the field (Warner, 2001).

In Germany, carabid species active during the period of pest larval drop included *A. dorsalis, P. cupreus, P. rufipes* and *P. melanarius*. A temporal and spatial association between these four species and *D. brassicae* was found using SADIE analysis (Felsmann & Büchs, 2003). However, a significant spatio-temporal association was restricted to the period with peak larval drop (16 - 23 May), and was not present for the duration of the experiment (22 April – 11 July). Further investigations appear to confirm the role of *A. dorsalis* and *P. rufipes* as important predators during larval drop. With the new knowledge about the feeding preferences of *A. similata*, this species is probably an important predator because of its dominant occurrence throughout the experiments.

In Poland, spatio-temporal studies are focussing on the stem-boring weevils, *C. pallidactylus* and *C. napi*. Early immigration of *C. pallidactylus* into a winter rape crop was predominantly by males, and distributions were at first edge-distributed and clustered. After 10-14 days, males became more centre-distributed but remained clustered. By contrast, later-arriving females were at first more loosely edge-distributed and less clustered and showed less tendency to become centre-distributed than the males. In *C. napi*, males were less clustered than the females.

Meligethes aeneus is conventionally controlled using pesticides, but pesticide use could be reduced by using trap crops to concentrate the pests. One potential strategy uses a trap crop of attractive early-flowering plants to protect the more susceptible late-bud stage of the main crop. In the UK, semi-field arrays of potted rape plants are being used to investigate this strategy, observing the development of distributions of *M. aeneus* in a simulated trap crop system (Frearson *et al*., 2004). In two experiments, total *M. aeneus* populations were counted on every plant, and total racemes in bud and racemes in flower were counted to assess host plant resources and cues. Inflorescence growth stage characteristics were shown to be important in determining the spatial distributions of *M. aeneus*. The beetles were usually spatially associated with plants with more racemes in bud and/or in flower. The trap crop maintained a significant edge distribution of *M. aeneus* for 7-10 days. While flowering racemes provided strong cues for immigrating *M. aeneus*, the abundance of buds was a more important determinant of residence time on the plants. In Germany, organic oilseed rape was grown with trap crop strips of the cultivar “Express”, a cultivar which proved to be attractive for most pests. In comparison to plots without trap crop at the margin, these trap crop strips showed a higher infestation by *M. aeneus, D. brassicae* and *C. assimilis* but not by *C. pallidactylus* (Büchs & Katzur, 2003). These experiments highlight the importance of establishing trap crops that are both attractive and provide resources that retain the pest.

Objective 2. To develop by field experiments economically-viable, environmentally-acceptable IPM strategies for European rape that maximise biological control of the target pests and minimise pesticide use.

Collaborative IPM strategies field experiments (Workpackage 1 led by Christer Nilsson) were initiated in autumn 2002 to compare two pest management systems for winter rape within a

cereal rotation and on a farm-scale, in five countries (Estonia, Germany, Poland, Sweden, UK): a Standard European Farming System (STN) and an Integrated Crop Management System (ICM). Each experiment is of two-years duration with insect monitoring in the winter rape crop and in the following winter wheat crop. Agreed protocols are being used for crop establishment and husbandry.

In 2002/3, there were three main treatment differences between STN and ICM plots. Firstly, soil tillage for the STN was by ploughing whereas that for the ICM was by non-inversion (minimal tillage) to improve conditions for epigeic predators. Secondly, although both plots were drilled with hybrid winter oilseed rape at 50 seeds per m^2, the seed used for the STN plots was cultivar Banjo alone, while that used for the ICM plots was a 98:2 mixture of Banjo and the turnip rape cultivar Salut. The turnip rape flowers earlier than the oilseed rape and is preferred by pests, thus acting as a trap crop. Thirdly, STN plots received 3 insecticide treatments whereas ICM plots received none. Data collection and evaluation of crop performance is by means of agreed indicators measuring yield and yield quality, use of production means, machinery and labour, plant density, pod damage and plant architecture, and incidence of stem diseases. Larvae of the target pests are collected and reared or dissected to assess the parasitisation levels and the incidence of pests. The insecticide treatments have partially reduced the pest larval populations, but have had only minor effects on the part of the populations attacked by parasitoids. The level of plant infestation by target pest species and the parasitism of larvae is being determined by taking repeated plant samples and dissecting pest larvae. The temporal coincidence of occurrence of above-ground predators, such as carabids, and the presence of pest eggs and larvae in the soil when they are most vulnerable to predation, is being monitored using pitfall traps.

In 2003/4, each STN and each ICM plot will be further divided. The STN and ICM plots will be treated in most respects as in 2002/3 but will differ in their insecticide treatments viz: STN-ii (intensive insecticide), STN-ie (insecticide to economic thresholds), ICM-ie (insecticide to economic thresholds), ICM-io (no insecticide). Following harvest of the oilseed rape crop, the seedbed for the winter wheat will be established by ploughing for the STN plot and by minimal cultivation for the ICM plot and parasitoid emergence on the plots compared.

To date, analysis of key predators from the ICM/STN collaborative experiments (pitfall trap samples) is restricted to carabids and the growing season of 2003. In Table 2, the partner countries involved are arranged in a geographical East-West gradient.

In all countries, except for Estonia, data is from winter rape; in Estonia they are from spring rape, because winter rape established poorly in the 2002 autumn drought and was destroyed by the severe winter. In winter rape, 14 carabid species achieved a dominance level of 5% or more in at least one partner country either in the STN or ICM system. There is no Europewide "standard" carabid community in oilseed rape fields, but each country shows a rather unique assemblage of species. In summary, the results show firstly, that the oilseed rape fields of each country (except Sweden) are inhabited by carabids which are dominant exclusively to that country (e.g. *N. brevicollis*, *P. madidus* and *A. flavipes* in the UK; *Stomis pumicatus* in Germany; *Harpalus brevicollis* in Poland). Secondly, for several species, extreme differences in the dominance level were recorded (e.g. *A. similata* 47.2% in Germany, but only 0.2% in Poland; *P. cupreus* 73.2% in Poland, but 2.5% in Sweden; *N. brevicollis* 29.8% in UK, but 2.7% in Germany and 0.0% in Poland). These differences were larger than the differences of dominance levels recorded in the ICM or STN within a partner country. Thirdly, the - phenological patterns of certain carabid species differed to a greater extent between the partner countries than that of the pests and their larvae. Generally, greater autumnal activity occurred in the Eastern (Poland, Estonia) than in the Western countries (UK, Germany).

Table 2. Carabid species dominant/subdominant in oilseed rape fields of the joint ICM/STN field experiment of UK, Germany, Sweden, Poland and Estonia in 2003. (all data from winter rape except Estonia where it is from spring rape).
Dominance **X=>**10%, X=>5%, x=>1%

	UK	Germany	Sweden	Poland	(Estonia)
Amara similata	**X**	**X**	**X**		
Anchomenus dorsalis	x	x	**X**		x
Asaphidion flavipes	X				
Bembidion lampros	x		**X**	X	
Calathus melanocephalus					**X**
Harpalus affinis	x	x	**X**	X	x
Harpalus brevicollis				X	
Loricera pilicornis	**X**	**X**	x		
Nebria brevicollis	**X**	x			
Notiophilus biguttatus	**X**	x			
Poecilus cupreus	X	X	x	**X**	X
Pseudoophonus rufipes	x	x	**X**	X	**X**
Pterostichus madidus	**X**				
Pterostichus melanarius	**X**	x	X	X	**X**
Stomis pumicatus		X			

Comparison of the carabid samples collected in the ICM and the STN systems showed that, in all partner countries, the average abundance both in the summer (May – July) and autumn (Sept. – October), and the number of species was greater overall in the ICM than in the STN system.

Objective 3. To determine the socio-economic feasibility, importance and economic efficiency of the IPM strategies in comparison with the current strategy of pest control in different countries of Europe, including gains in environmental quality and rural viability, and to assess the socio-economic factors influencing their adoption.

This objective is the remit of Workpackage 5: Socio-economics led by Ingeborg Menzler-Hokkanen. A Europe-wide survey of oilseed rape farmers (1500 in six countries) is being conducted in 2004. This aims to find out about their attitudes to different pest management systems, how and why they adopt IPM systems and how they evaluate cost/benefits. The questions (24 in total, but most with a set of sub-questions) are designed to give information on current pest management practices, the decision-making involved in it, farmers' awareness of various IPM components and their potential role in pest management, and on factors which hinder or encourage farmers to adopt IPM practices.

A pilot study with responses from 25 farmers in Finland and 15 in Estonia has already been conducted. The indications from this study are very interesting. Current practices and the associated decision-making cover a broad range of alternatives. For example, each of the nine alternatives offered as a basis for deciding whether to spray with insecticides or not are being used by some farmers, including spraying by calendar date. Most farmers suspected that pest pressure on the oilseed crop is influenced by factors such as crop rotation, neighbouring

crops, or proximity to uncultivated land, while most acknowledged having no idea whether soil tillage, predatory or parasitic insects, or insect pathogens influence pest populations. This indicates a clear need for educating farmers about the beneficial role of natural enemies in pest control. Questions addressing the farmers' willingness to change their pest management system yielded rather straightforward replies: both the 'carrot' and the 'stick' approach would work. If the economics of farming improves, there is willingness to change husbandry practices. However, some intriguing patterns emerged too; willingness to adopt IPM practices was dampened should pest problems increase after adoption, even if the use of insecticides were drastically cut, and profit remained the same or even increased.

The full survey should give an invaluable overall picture of rapeseed growers' attitudes in Europe towards pest management and IPM. Analysis of responses will also indicate constraints to implementation of any IPM strategy proposed by this project for growing oilseed rape in Europe, and what kind of policy measures might be required to encourage adoption of IPM practices at the farm level.

Objective 4. To construct a Phenological Model of target pests and their bio-control agents, that relates their times of occurrence and activity on the crop to growth stage and climatic/weather conditions, for integration into existing Decision Support systems.

The phenologies of the occurrence, flight and activity of the target pests and their key parasitoids to and on the crop are being monitored each year by means of yellow water traps placed at canopy height in the crop in five partner countries (UK, Sweden, Estonia, Germany and Poland). This information will be related to vulnerable crop growth stages and to climatic/weather conditions. It will help to define spray windows compatible with natural enemy conservation for integration into the computer-based decision support system proPlant (Johnen & Meier, 2000). This system already has pest Phenological Models for the six target pests of this project, based on eight years of field observations, on the influence of the weather on their population dynamics in different regions of Germany. The program takes into account numbers of adult pests, weather-based forecasts of flight conditions, egg-laying periods and larval development. The models automatically collect regional meteorological data via internet or home-run meteorological stations to predict pest infestation and the need for control.

Objective 5. To produce Technical Guidelines on the IPM strategies for farmers, advisors and policy makers.

Project results will be made widely available through scientific and extension publications, the project website: www.iacr.bbsrc.ac.uk/pie/master/master.htm, the PC-based computer decision support system proPlant, technical guidelines for growers and a dedicated workshop at the end of the project.

Technical Guidelines on the IPM Strategies will be produced in the form of practical, on-farm, management measures that will enhance the positive effects of the key bio-control agents and minimise pesticide use while maximising cost effectiveness of crop production. Analyses of grower views will aid the drafting of these Guidelines. In the final year of the project, a dedicated Symposium will be organised by the British Crop Production Council (BCPC) to disseminate further the results of the project and to provide a forum for discussion with end-users. Its venue and timing will be announced on the website, but provisionally has been set for April 2006 in Goettingen, Germany.

Discussion

The management of pests on the European winter oilseed rape crop still relies heavily on chemical pesticides, most often applied routinely and prophylactically, often without regard to pest incidence, and at best, according to threshold values of the pest population. Over-use of chemical pesticides reduces the economic competitiveness of the crop, threatens biological diversity and risks selecting for resistance by target pests. Pesticides may also kill the natural agents of biological control, which would otherwise be a natural resource of great potential benefit to the farmer and consumer. This project aims to utilise recent advances in our knowledge of the ecology of the pests, their natural enemies and the effects of husbandry practices on the invertebrate fauna (Williams, 2004) into IPM strategies for winter rape to improve the efficiency, profitability and environmental acceptability of production and, thereby, contribute towards sustainable production of the crop.

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SECURE – possibilities for durable resistance to stem canker?

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Abstract: In many European countries, phoma stem canker (*Leptosphaeria maculans*) control is currently reliant on the use of foliar fungicide sprays. Although the use of resistant cultivars can offer a viable alternative, the pathogen is highly variable and single-gene resistance tends to break down within a few seasons due to the emergence of new races after the widespread release of resistant cultivars. The SECURE project (StEm Canker of oilseed rape: molecular tools and mathematical modelling to deploy dUrable REsistance; QLK5-CT-2002-01813) aims to deliver a model for deployment of cultivars with resistance to *L. maculans* to improve durability of resistance. There are four targeted specialist groups of end users: policy makers/politicians/government agencies; plant breeders / advisers / farmers; the international scientific community; citizens groups and the general public. An important mechanism for delivery is the SECURE web-site (www.secure.rothamsted.ac.uk). Other more traditional routes of dissemination are press articles, field demonstrations, workshops, breeding programmes, conferences and peer-reviewed refereed papers.

Key words: *Leptosphaeria maculans*, *Phoma lingam*, disease resistance, molecular biology, mathematical modelling.

Introduction

Leptosphaeria maculans (anamorph *Phoma lingam*) causes phoma stem canker (blackleg), an oilseed rape (*Brassica napus*) disease of major economic importance in Europe, Australia and Canada. In many European countries, disease control is currently reliant on the use of foliar fungicide sprays applied during the phoma leaf spotting phase of the epidemic. Although resistant cultivars have provided acceptable levels of control against the disease in the past, the pathogen is highly variable and single-gene resistance tends to break down within a few seasons due to the emergence of new races (Brun *et al*., 2000). The EU funded SECURE project (StEm Canker of oilseed rape: molecular tools and mathematical modelling to deploy dUrable REsistance; QLK5-CT-2002-01813) aims to deliver a model for deployment of cultivars with resistance to *L. maculans* to improve durability of resistance and minimise risk that the resistance will break down.

Material and methods

Objectives of SECURE

There are four main objectives within the SECURE project:

1. To construct a model of the life cycle of *L. maculans* and validate it with existing data.
2. To compare the fitness of virulent/avirulent races of the pathogen and develop genomic analysis of avirulence and virulence loci.
3. To analyse effects of plant genetic background and environmental factors on durability of resistance, in both field and controlled conditions.
4. To model effects of resistance deployment strategies on durability of resistance and recommend a sustainable strategy.

The project has five workpackages (WP). The first four WP's reflect the four main scientific objectives of the project whilst the fifth WP concerns project management and the dissemination of information and recommendations to the target groups of end users.

***WP 1: Modelling the life cycle of* Leptosphaeria maculans**

A mathematical model of the *L. maculans* life cycle on winter oilseed rape is being constructed, and values of the parameters estimated from extensive data sets. The model is based on the important stages in the life cycle of *L. maculans* on winter oilseed rape in Europe. These are (i) ascospore production, (ii) infection of leaves, (iii) leaf phoma development, (iv) growth of the fungus through the petiole and (v) stem canker development. The model incorporates effects of meteorological variables (leaf wetness duration, temperature) on the various stages of the life cycle.

The model will initially be tested qualitatively using existing data sets. Additional data for model testing ("validation") will include data from controlled environment experiments on stages in the life cycle and new data on phoma leaf spot and stem canker development from field experiments in WP 3.

WP 2: Effects of pathogen variation at Avr loci on durability of resistance

Work is analysing, at the molecular level, allelic variation at three avirulence loci. Chromosome walking techniques and ultimately cloning will allow analysis of the mutational events causing loss of avirulence function ("gain in virulence"). Other studies compare the fitness of avirulent vs. virulent races of *L. maculans* under controlled environment and field conditions using near isogenic lines of the pathogen which differ only at the *AvrLm1*, *AvrLm4* or *AvrLm6* locus.

WP 3: Effects of genotype/environment on durability of resistance

The work is characterising some effects of genotype and environment on durability of resistance. For example, a novel inoculation method has been developed (Huang *et al*., 2004) and this is being used to investigate the influence of plant genetic background and environmental variables (temperature and leaf wetness) on durability of resistance (Olechnowicz *et al*., 2004). Plants with the major resistance gene *Rlm6* in different *B. napus* genetic backgrounds (cv. Eurol, susceptible; cv. Darmor, polygenic resistance) are being used.

Field work includes an initial sample of the *L. maculans* population throughout the main oilseed rape growing regions of Europe (Figure 1). Preliminary results are published in this volume (Balesdent *et al*., 2004; Stachowiak *et al*., 2004). Other field work will assess durability of a number of commercial cultivars and two *Brassica napus* lines under field conditions at a number of sites across Europe (Fig. 1a). The material includes cv. Darmor (one known resistance gene *RLm9* and polygenic resistance efficient at the adult stage) and

cv. Eurol (two known resistance genes *RLm2 and RLm3*) and two lines named DarmorMX and EurolMX which consist of the same two parent cultivars with the addition of the novel resistance gene *RLm6* introgressed from *Brassica juncea*.

WP 4: Strategy for sustainable deployment of durable resistance

Using results/data from WP1, WP2 and WP3, a mechanistic model to study effects of deployment strategy on durability of resistance (using and comparing new criteria) is being produced to guide sustainable deployment of durable resistance to stem canker in Europe (van den Bosch and Gilligan, 2003; Pietravalle *et al*., 2004).

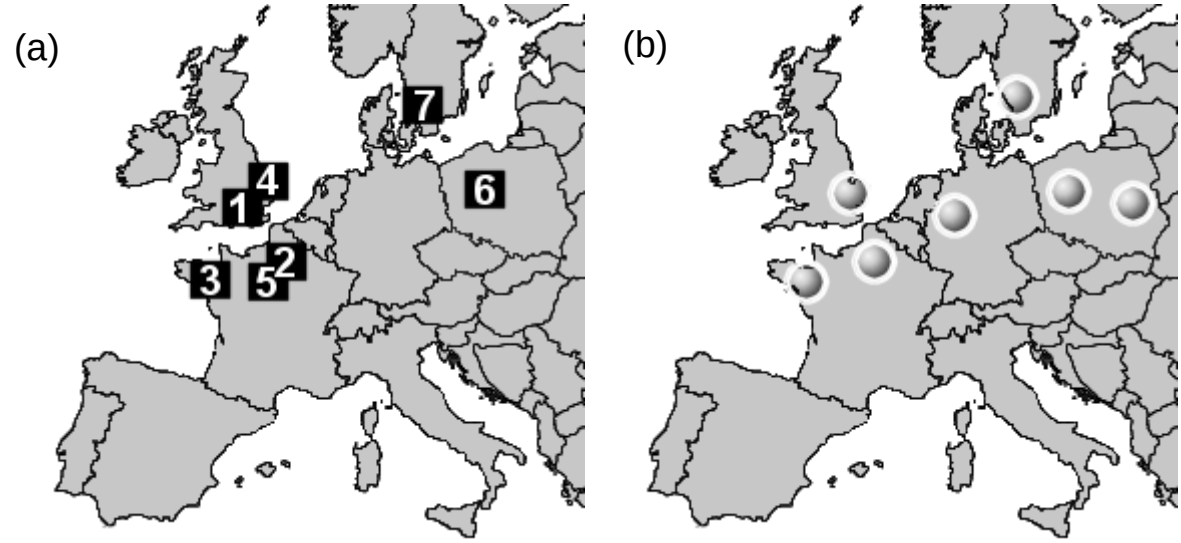

Figure 1. Maps indicating the location of SECURE partner organisations (a) and experimental field sites (b) throughout the main oilseed rape growing regions of Europe. Partner numbers relate to the project partner number: P1 - Rothamsted Research, UK; P2 - Unité Phytopathologie et méthodologies de la détection (PMDV), INRA Versailles, France; P3 - Unité Mixte de Recherche INRA-ENSAR Biologie des Organismes et des Populations appliquée à la Protection des Plantes (BiO3P), INRA Rennes, France; P4 – ADAS Boxworth, UK; P5 – CETIOM, France; P6 - Instytut Genetyki Roslin, Polska Akademia Nauk [Institute of Plant Genetics, Polish Academy of Sciences], Poland; P7 - Svalöf Weibull AB, Sweden.

WP 5: Management of project and dissemination of results.

This WP concerns project management and delivery of results, notably new models and recommendations to users. There are four targeted specialist groups of end users (A. policy makers / politicians / government agencies; B. plant breeders / advisers / farmers; C. the international scientific community; D. citizens groups and the general public). An important mechanism for delivery is the SECURE web-site (www.secure.rothamsted.ac.uk) (Fig. 2). Other routes of dissemination are traditional routes such as press articles, field demonstrations, workshops, breeding programmes, conferences and, importantly, peer-reviewed refereed papers.

Figure 2. SECURE project website homepage giving details of the aims of the project and links to other areas of the SECURE website, including websites of all research and industrial partners.

SECURE partner organisations and the role of each partner in the project

There are seven main partners in SECURE (Fig. 1a). The project participants include many of the major research organisations and extension services involved with phoma stem canker on oilseed rape in Europe. Partner 1 is Rothamsted Research, UK, which will coordinate the project on behalf of the consortium. Work at Rothamsted Research is using controlled environment facilities to do work on effects of environmental parameters on resistance responses. In addition, P1 is leading the modelling work done in WP1 and WP4, as the majority of the work is being done by the Mathematical Modellers Group of the Biomathematics Unit at Rothamsted Research. Partner 2 is the Unité Phytopathologie et méthodologies de la détection (PMDV), INRA Versailles, France. Work at Versailles concerns study of the effects of pathogen variation at Avr loci on durability of resistance. P2

leads WP2. Partner 3, Unité Mixte de Recherche INRA-ENSAR Biologie des Organismes et des Populations appliquée à la Protection des Plantes (BiO3P), INRA Rennes, France is working in a wide variety of disciplines in WP2 and WP3 and leads WP3. Partner 4, ADAS Boxworth, UK is doing field experimentation in WP3 and uses its near-market / near-user position to lead dissemination activities in WP5. Similarly, partner 5, CETIOM, France is doing field experimentation under WP3 and provides the primary route of dissemination to the French oilseed rape industry. Partner 6, the Instytut Genetyki Roslin, Polska Akademia Nauk [Institute of Plant Genetics, Polish Academy of Sciences], Poznan, Poland is doing experiments in WP1, WP2 and WP3. P6 is doing molecular work and fitness studies in WP2, field evaluation and controlled environment work in WP1 and WP3. P6 is responsible for disseminating outcomes from SECURE through the Concerted Action project PAGEN. Partner 7, Svalöf Weibull AB, Sweden, a plant breeding company with breeding interests throughout Europe, is responsible for field experimentation in Sweden and Germany (WP3) and involved in dissemination activities in both of these countries (WP5).

Acknowledgements

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Diseases / Pests: Decision models and plant protection measures

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Effects of conservation tillage on harmful organisms and yield of oilseed rape

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Abstract: In a long-term field trial, the effects of three different tillage systems on harmful organisms and yield were investigated. The focus was on fungal diseases, weeds and slugs. With the ploughing system as the standard, a non-inversion/ conservation tillage and a direct drilling/no till system were compared with one another. The crop rotation oilseed rape-wheat-barley, which was established in 1995, was reconverted into a crop rotation oilseed rape-wheat-wheat in 1998 due to problems with volunteer wheat in the following barley in the two ploughless tillage systems. The occurrence of Phoma root-collar and stem disease, the most important in Germany, was not affected in comparison over the years by the intensity of the cultivation. For Sclerotinia stem rot, a correlation could only be determined with the tillage systems in one year of the trial series. The infection became more severe with decreasing intensity of soil cultivation. Whether this result can be reproduced in future growing seasons remains to be seen. Effects on the incidence of *Verticillium longisporum* could not be determined. Other diseases arose only sporadically at very low levels. However, in comparison, the occurrence of weeds was affected significantly. The amount of grass weed species (*Alopecurus myosuroides*, *Apera spica-venti*, volunteer barley) increased in the systems without ploughing. The effect on dicotyledonous weed species was dependent on the particular species. In individual years, heavy slug damage could be correlated with direct drilling system.

Key words: oilseed rape, tillage system, canker, *Leptosphaeria maculans* anamorph: *Phoma lingam*, diseases, weed control, slugs

Introduction

In Germany, conservation tillage systems are becoming more important in agricultural practice. This applies to non-tillage systems only in special cases. Reasons to carry out conservation tillage are to lower labour and machinery costs, protect against erosion, increase the load-bearing capacity of the soil and to support soil organisms. In some regions in Germany there is an additional financial support for these tillage systems. Effects on harmful organisms due to soil tillage are known from North America and the UK. Problems were particularly described for the cultivation of grain (Brooks & Dawson 1968, Boosalis & Doupnik 1976, Prew et al. 1995). An increase of certain monocotyledonous weeds in Germany was also reported (Knab 1988, Arnold-Reimer 1994, Sievert 2000, Kreye 2002). Some diseases increase under such systems, particularly those that develop on crop debris on the soil-surface. Of all the rapeseed diseases, this is particularly true for Phoma. Unfortunately, only a short period is often examined in most investigations. For this reason a long-term field trial, with different tillage systems, was begun in 1995 to investigate the effect of crop rotation on harmful organisms of winter oilseed rape.

Materials and methods

A field trial was established at Sickte, near Braunschweig, on soils ranging between sandy loam and loamy clay. The area had an average rainfall of 625mm and a mean temperature of 9.6°C. The lay-out of the field trial was a split-plot design with two repetitions. Three types of soil cultivation were tested: ploughing as the standard (25-30 cm deep), conservation tillage by grubbing (15-20 cm deep) and no till/direct drilling. After we had a lot of problems with volunteer wheat in the following barley, we changed the crop rotation oilseed rape-wheat-barley, established in 1995, to oilseed rape-wheat-wheat in 1998. Each crop was cultivated each year in each cultivation system. The plots were managed under normal agricultural practices. The stubble breaking was done with a heavy cultivator twice in the ploughing and the noninversion tillage system. After primary tillage, the sowing was done by a rotary harrow and a seed drill with disc coulters in both systems. In the direct drilling system we only used a straw curry-comb and sowed using a direct seed drill with chisel coulters. All plots received an application of nitrogen in the autumn (30-40 kg N/ha). In the direct drilling systems, there was an application of herbicide before sowing. In the conservation tillage system, herbicide application only took place if further weeds developed. Assessments of canker caused by *Phoma lingam* were made at growth stage 81 (BBCH) just before harvest. We used the scheme of Krüger (1983) to estimate canker symptoms at the root collar. To assess sclerotinia stem rot, the frequency of damaged plants was determined at growth stage 79 (BBCH). An assessment of the weed population was done by identification of species and a count of the total number of each species. Additionally, we estimated the weed and crop coverage of the soil surface. To estimate slug infestation incidence in soil and damage to oilseed rape we used refuge traps (Bayer Schneckenmatte) and areas with or without molluscicide treatment after drilling. Since 2002 we tested a modified flooding method for soil samples with other partners (GLEN *et al.*, 1992).

Results and discussion

On the basis of the life cycle of *Leptosphaeria maculans*, a fair assumption might be that oilseed rape epidemics might be expected to increase with noninversion tillage or direct drilling. Infected rape residues remain on the soil surface and ascospores can be spread by the wind to nearby fields with recently emerged oilseed rape. Figure 1 shows the infestation values in the individual tillage systems over the years. However, there was no uniform effect due to the tillage systems. Because oilseed rape after oilseed rape is not usually cultivated, the influence of the mobility of the ascospores on the infection of emerged rape plants must be a major factor for this effect. This effect is independent of the tillage system, because even in the ploughed system, rape residues are left in the field until Mid-September. However, incorporation of rape residues through all tillage practices decreases the potential inoculum. Because of the importance of ascospores and their mobility for the infection of rape plants, it is probably the concentration of rape cultivation in an area that is important.

The effects on sclerotinia stem rot were different. In 2002, at the beginning of the third rotation we found an increased level of infection due to tillage. Infection became more severe with decreasing intensity of soil cultivation (Fig. 2). However, this effect was detected only once to date. In the other years the levels of infection were low and there were no significant differences. This indicates that the influence of the weather on the infections was more important. It cannot be answered whether the presence of sclerotia in the top soil layer leads to increased infection. It is possible that there is a higher germination rate of sclerotia in the

ploughless tillage systems due to their presence in the top soil. The absence of a host plant at the time of sclerotia germination in the interval of cultivation leads to a lower number of sclerotia in the following oilseed rape. It must be seen if the result of 2002 is repeatable in future growing seasons.

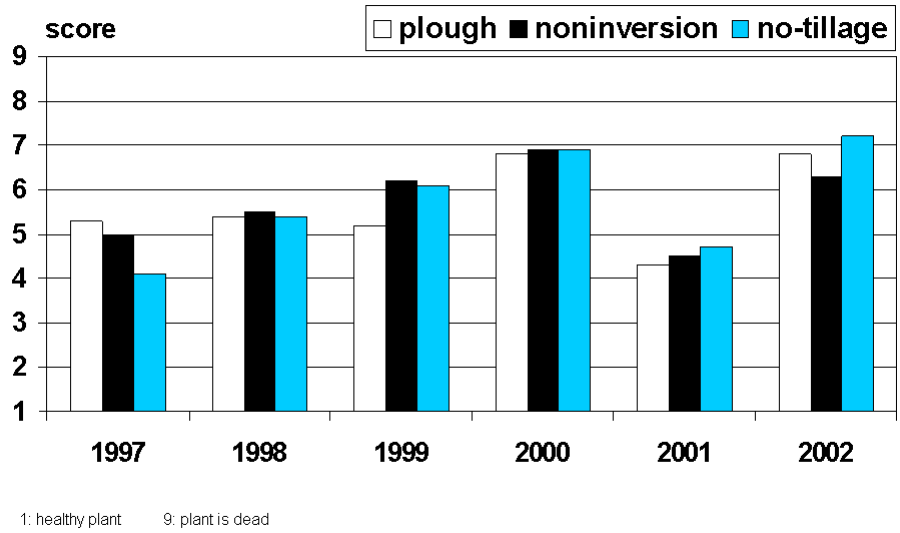

Figure 1: Phoma root-collar infection on oilseed rape in different tillage systems, BBCH 81

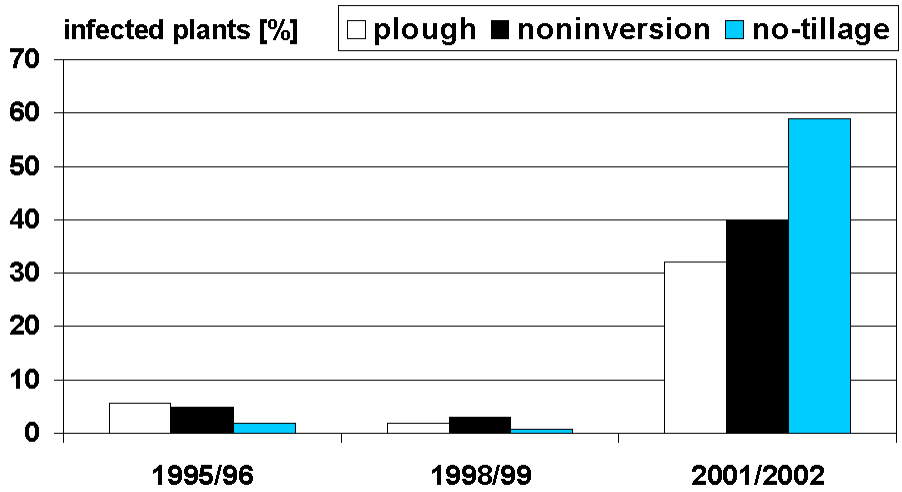

Figure 2: Infection of oilseed rape by *Sclerotinia sclerotiorum* without fungicide treatment at flowering stage

Until now the infection frequency of Verticillium stem disease was very low (<1%) and not influenced by the tillage systems. Observations in Germany indicating an increase of the infection of oilseed rape by *Verticillium longisporum* with noninversion tillage or direct

drilling have not be proved up to date. Other diseases were found only occasionally and they were also not influenced by the tillage systems.

Effects on the weed population in oilseed rape were already found at the beginning of the experiments. This is particularly true with respect to the amount of grass weed plants (*Alopecurus myosuroides*, *Apera spica-venti*, volunteer barley) which increased in the systems without ploughing (Sievert 2000). Sometimes there were fewer grass weeds per m² in the direct drilling plots than in ploughing in autumn and more spikes of *A. myosiroides* left at harvest. This effect is due to gaps in the oilseed rape population. At these sites, the oilseed rape does not suppress the growth of weeds. Also some dicotyledons profit from this effect. But the higher amount of weeds in spring, which is illustrated in figure 3, might be due to a decreased efficacy of herbicides. It is possible that an active ingredient is fixed at the residues at the soil surface.

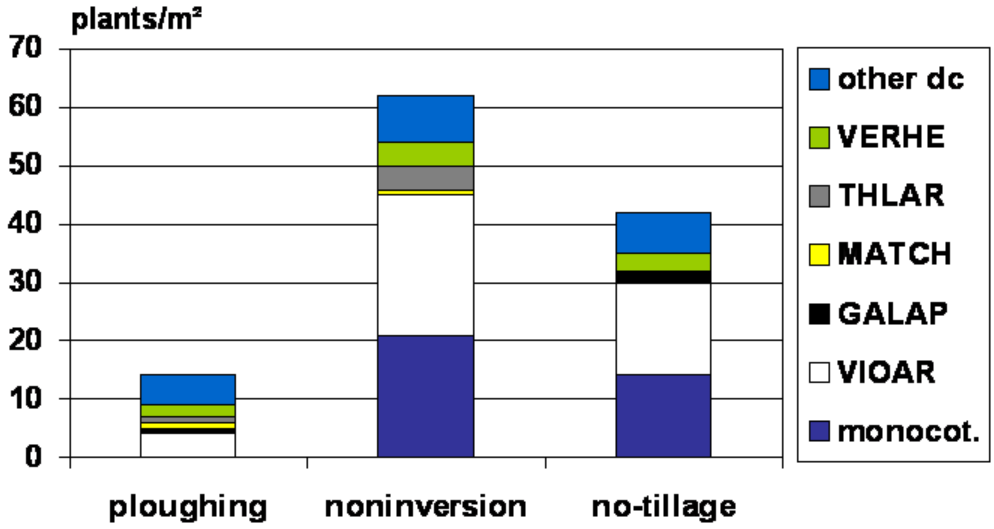

Figure 3: Weeds left in spring 2004 after herbicide application in summer 2003

The number of slugs increased without ploughing. This is comparable with results from Voss et al. (1998). Especially in direct drilled plots, there was severe damage due to slugs. In the extreme case of 2002, there was no oilseed rape left in late autumn. However, slugs were not a problem every year. In the conservation tillage system the situation was less severe. Tillage at the right time is an effective method to reduce the number of slugs.

Beside the problems with slugs there was also damage by field mice in the two less intensive tillage systems. These harmful organisms profited by the longer soil peace. Mouse damage had the added effect of increasing the density and development of the weeds.

It was potentially possible to obtain comparable yields in the three different tillage systems. On the average of the years, there was no difference between the standard tillage system “plough“ and the noninversion treatment. In the less intensive tillage system, yields obtained were higher, but not significantly so. However, the yields of the direct seed varied more, as is shown in figure 4. It is evident that for obtaining higher yields, tillage is not inevitably necessary, but it decreases the risks from diseases and/or weeds.

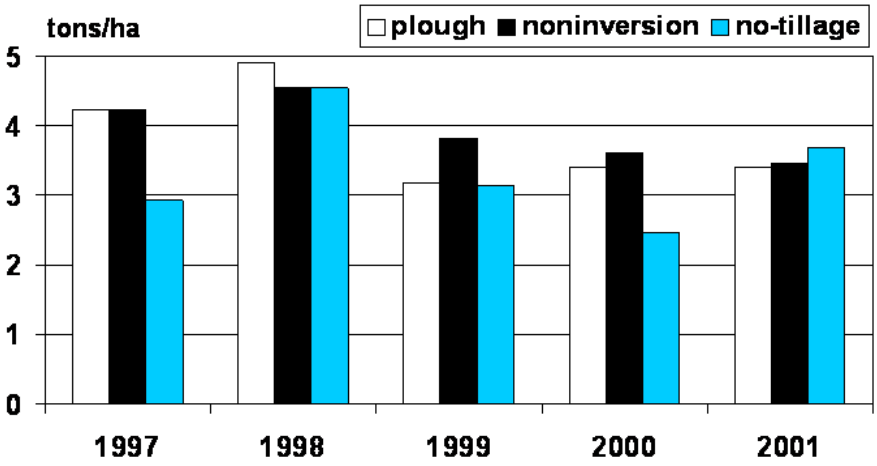

Figure 4: Yield of oilseed rape in different tillage-systems

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The PASSWORD project: a decision support system for managing pests and diseases of winter oilseed rape in the UK

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Abstract: Pests and diseases of winter oilseed rape are estimated to cause losses of up to £80 million per annum in the UK. Pesticides are widely used to control them, but treatments are not always used effectively. Decision-making is complex for farmers because of the large seasonal and regional variation in the occurrence of pests and diseases. These issues have been addressed through a project to develop a decision support system known as PASSWORD. This was initiated in October 2000 and continues with a model testing phase up to September 2005. The PASSWORD project has combined a decision support system for the major invertebrate pests of oilseed rape (known as DORIS) developed by CSL, York, with new disease components for the most important diseases, phoma stem canker (*Leptosphaeria maculans*) and light leaf spot (*Pyrenopeziza brassicae*). Data generated from pest and disease surveys, biological studies and field experiments have been used to develop disease forecasts at the regional level and models for disease development and yield loss at the crop level. During the growing season, daily weather data and current crop information are required to run the models and to determine what action is required. The models were developed through close consultation with industry representatives at every stage of DSS construction and are being validated using a range of techniques.

Key words: decision making, invertebrate pests, light leaf spot, phoma leaf spot, stem canker

Introduction

Oilseed rape is the most widely grown break crop in the UK, with annual production of about 400,000ha. This could increase in future under the reformed Common Agricultural Policy, if the crop can be grown profitably. Pesticides are used on most crops and in 2002, 80% crops received insecticides and 88% crops received fungicides (Garthwaite et al., 2003). Effective use of these treatments is important for sustainable production and for protection of the environment. However, surveys of diseases and pests indicate that they are still causing yield losses estimated at up to £80 million/annum (Fitt et al., 1997). Some pesticide treatments are ineffective because of poorly timed applications or unnecessary because the target pest did not cause damage. The decision-making process for pests and disease control is complex, particularly because the occurrence of pests and diseases varies from year to year and crop to crop. Better guidance is required by farmers and advisers to improve targeting of pesticide

treatments. The PASSWORD project was undertaken to exploit and extend existing biological understanding of pests and diseases and their interaction with the crop and to deliver a computer-based system to guide decisions on farms.

Methods

The PASSWORD project (Pest and disease mAnagement System Supporting Winter Oilseed Rape Decisions) started in October 2000 with industry (Home-Grown Cereals Authority, DuPont (UK) Ltd, Syngenta Crop Protection Ltd, The ProCam Group and the Perry Foundation) and Government funding through the Sustainable Arable LINK programme. Research partners are ADAS, the Central Science Laboratory, Rothamsted Research and the Scottish Agricultural College. The first phase of the project was completed in September 2003 and a second two year phase to test new disease models is now in progress.

The project integrated existing Decision Support System for pests, DORIS (Decision support Oilseed Rape Invertebrate pestS) and MOPI (Management of Oilseed rape Pests via the Internet) developed at the Central Science Laboratory (Morgan et al., 1998), with new research on stem canker (*Leptosphaeria maculans*) and light leaf spot (*Pyrenopeziza brassicae*) in winter oilseed rape. The pests included are aphids as virus vectors (*Myzus persicae*), cabbage stem flea beetle (*Psylliodes chrysocephala*), rape winter stem weevil (*Ceutorhynchus picitarsis*), pollen beetles (*Meligethes* spp.), cabbage seed weevil (*Ceutorhynchus assimilis*), brassica pod midge (*Dasineura brassicae*) and summer aphid outbreaks (*Brevicoryne brassicae*). Data from Defra-funded national disease surveys, various replicated fungicide and cultivar x fungicide experiments and epidemiological studies were available to develop new disease models.

Results

Light leaf spot regional forecast

A regional forecast for light leaf spot developed prior to this project has been produced each autumn. The most important factors in the light leaf spot forecast are the incidence of light leaf spot on pods pre-harvest (inoculum) and summer temperature deviation from the long term mean. The forecast predicts the percentage of crops that are likely to show 25% or more plants with light leaf spot at the early stem extension stage (in March). An Internet-based interactive version of the forecast has been developed that allows users to provide estimates of the light leaf spot incidence in their own crops by identifying their cultivar, date of sowing and any autumn fungicide use (http://www3.res.bbsrc.ac.uk/leafspot). An update of the light leaf spot is available in February to take account of actual autumn rainfall. The reliability of the forecast has been tested using new disease survey data from farm crops and predicted values were reliable in 86% of cases during 2000-2003.

Phoma stem canker regional forecast

A new regional forecast for phoma stem canker incidence pre-harvest has been developed within the PASSWORD using a similar approach to that used to develop the light leaf spot forecast. Previous disease and monthly temperature and rainfall data were the most influential factors. The phoma regional forecast is produced in autumn and predicts the regional incidence of stem canker pre-harvest (late June) using long term weather data. The phoma forecast has been made available on the Internet (http://phoma.csl.gov.uk). Users can also

access details of the life cycle, historic survey data and register to receive updates of the forecast (Fig. 1). Updates are produced in spring when actual autumn and winter weather records are available.

Both the phoma stem canker and light leaf spot forecasts are strategic tools, enabling users to make more informed decisions about fungicide use. The interactive format allows different scenarios to be played to evaluate the influence of changing cultivar, sow date or autumn fungicide use.

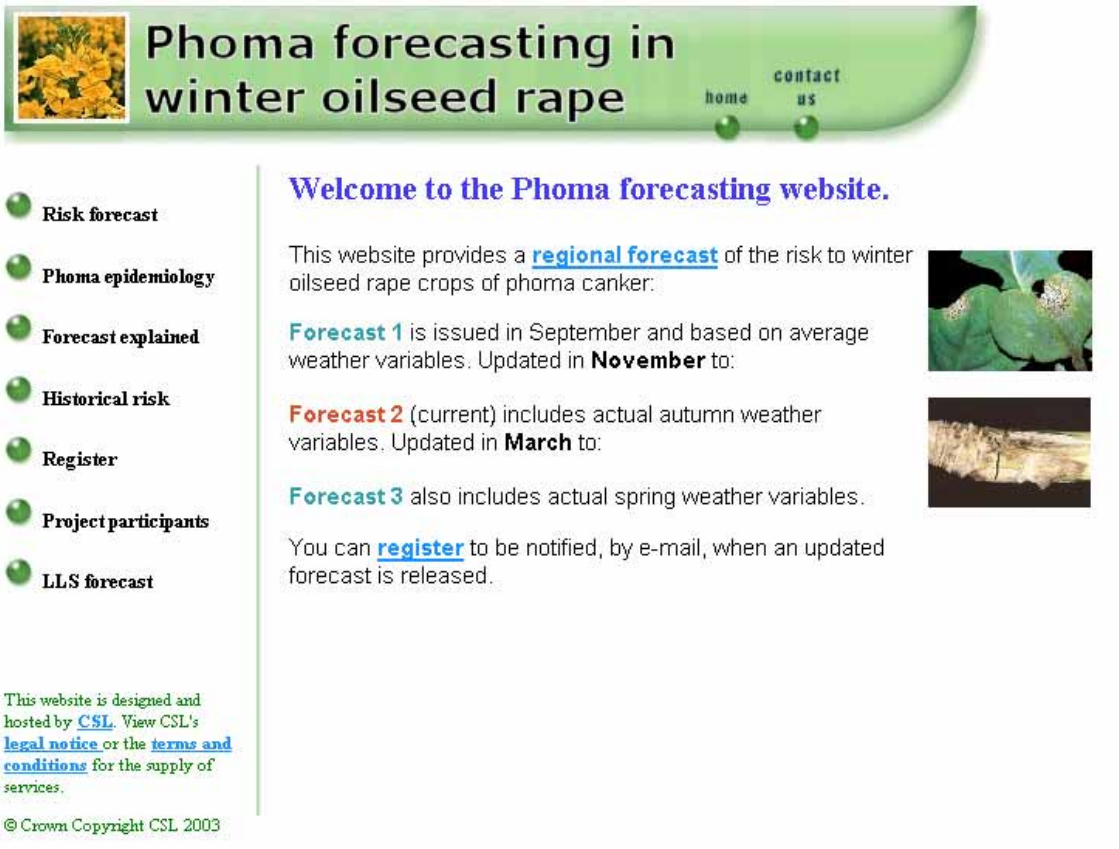

Figure1. Home page of the Phoma regional forecast.

Crop risk models

Crop risk modelling had been undertaken for both light leaf spot and phoma leaf spot/stem canker. A four stage model for phoma stem canker predicts (1) the date when 10% plants will have phoma leaf spot (2) the time between appearance of phoma leaf spot and appearance of stem canker (3) the increase in stem canker severity from first appearance up to harvest and (4) the yield loss in relation to canker severity. The timing of the onset of phoma leaf spotting in autumn is strongly influenced by rainfall between harvest and late September. Dry conditions delay the maturation of pseudothecia on crop residues, whilst high rainfall in August and September leads to early maturation of pseudothecia and hence early symptoms of phoma leaf spot. Early phoma leaf spot epidemics lead to early development of stem canker symptoms in spring and these become progressively more severe up to harvest. Yield loss is associated with stem canker lesions which affect >50% stem circumference pre-harvest (Zhou et al., 1999).

Cultivar resistance influences both the thermal time period between the appearance of phoma leaf spot and the appearance of stem canker and also the increase in canker severity

over time. Fungicides produce a similar, though often larger effect, than can be achieved by increasing cultivar resistance. Both cultivar resistance and fungicides can produce yield benefits by delaying the development of stem canker. Two sprays of a triazole-based fungicide programme applied in the autumn at onset of phoma leaf spot with a second spray 6-8 weeks later has provided more reliable disease control and more consistent yield responses than single spray treatments. In high risk areas, autumn and winter fungicides also provide cost-effective control of light leaf spot. Selected experimental datasets are available within PASSWORD that may be used to explore how individual fungicide applications or combinations of sprays contribute to disease control and yield responses with differently timed epidemics.

Integration of pest and disease decisions

The existing DORIS DSS for invertebrate pests has already undergone validation and is being made available for use from autumn 2004. The integration of pest and disease decisions forms a novel part of the PASSWORD system, offering some savings in treatment application costs where fungicide and insecticide treatments can be combined.

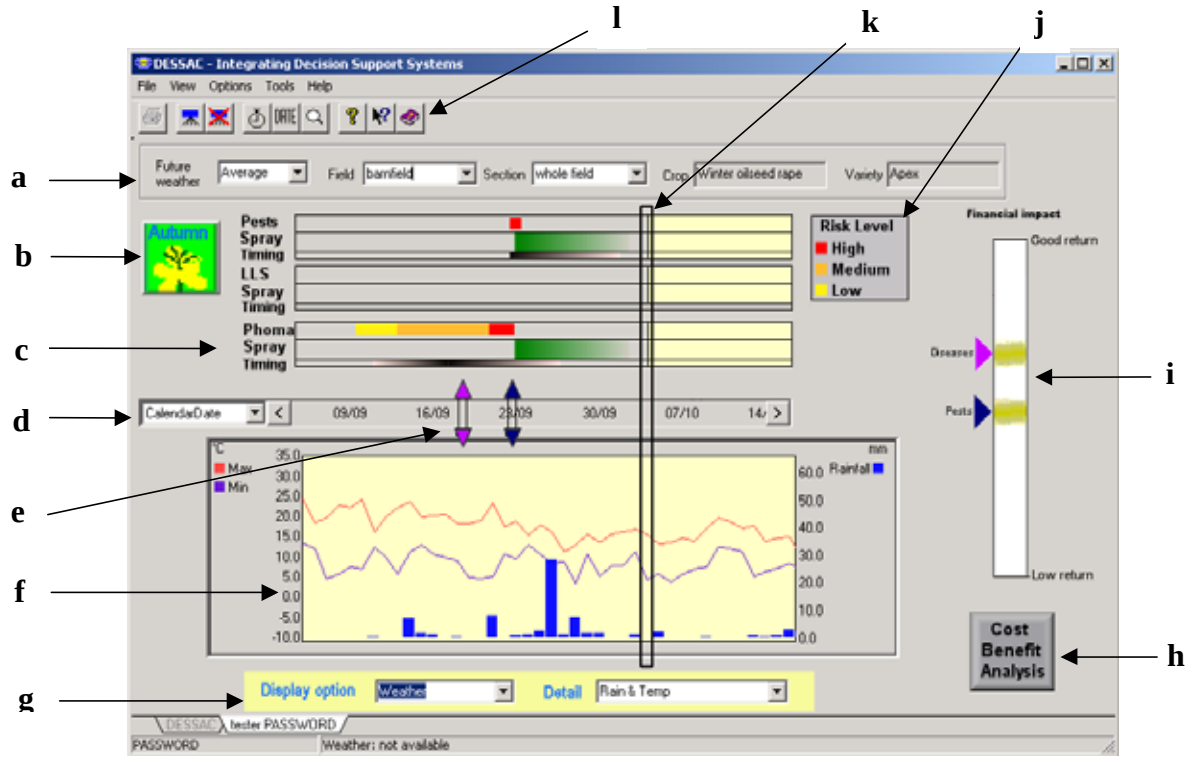

Figure 2. Screenshot of the main interface of the PASSWORD module. (a – l are described below).

Interface description (from Fig. 2)

a) Farm Bar: Contains the information on, and allows changes between, the fields and field sections on the current farm. Also allows the user to change the weather data used in the future predictions of disease.

b) Model run button: Clicking on this button allows the user to enter information on the latest pest sampling. The disease models run automatically on starting the module.

c) Risk bars: Pests and diseases: There are three sets of risk bars in the interface, one set for the pests (dealt with holistically) and a separate set for light leaf spot and phoma stem canker. Each set contains three bars – 'risk', 'spray' and 'timing'.
d) Timeline: Shows the timescale that is currently on view.
e) Treatment markers: These highlight the models recommended timing of fungicide (pink) and insecticide (blue) treatments. These markers can be dragged along the time line enabling the visualisation of the financial impact of treating earlier or later.
f) Display area: This area contains the information chosen via the display options. Choices include a variety of weather data and historical pest or disease incidence.
g) Display options: These drop down boxes allow the user to choose what is to be displayed in the display area.
h) Cost benefit analysis: Clicking on this button will display a pop-up box containing estimates of the financial effects of the disease levels and current treatment regime.
i) Financial impact bar: This allows the user to visualise the relative effects of the pests and diseases on the overall return from their crop.
j) Key: The key refers to the level of risk associated with the pests and diseases.
k) Date indicator: This indicates today's date on the display.
l) Toolbar: The various buttons on the toolbar provide access to the help files and encyclopaedic information, functions to change the date in view and the scope of the display.

User requirements

A number of workshops have been organised to collate views from potential users (e.g. farmers, consultants, agrochemical companies) on priorities for DSS, design of the interface and delivery systems. The project has addressed priority areas for support, providing early warning of disease outbreaks, optimal and sub-optimal use of pesticides, economic appraisal of decisions and encyclopaedic information on life cycles. Some priorities identified by potential users were outside the scope of the current project and included guidance on sclerotinia stem rot (*Sclerotinia sclerotiorum*) control. The interface design (Fig. 2) shared many common features with that used by Arable DS and was well received. Most users considered that the system for data entry was easy to use. Photographic images of pests and diseases were requested to support field diagnosis and action thresholds. Users differed in their preferences for communication of results. Whilst some favoured Internet or PC-based systems, many farmers had a preference for e-mail warnings or faxes and these are now available to users who request updates.

Commercial development

PASSWORD has been developed as a potential module for the DESSAC (now Arable DS) decision support system (Brooks, 1998). Arable DS offers a framework of software and commonly used data on data links including weather data, pesticides and farm crop records. Arable DS currently has a `Wheat Disease Manager` module that was launched in 2003 and its use is being extended in 2004. PASSWORD will be available from autumn 2006 (subject to satisfactory testing in 2004 and 2005) for field evaluation and commercial development.

Discussion

Developments with the PASSWORD project have allowed farmers and advisers to access disease forecasts for stem canker and light leaf spot via the Internet. New models predicting the appearance of phoma leaf spot and stem canker symptoms and subsequent disease

progress will allow the risk of yield loss to be quantified more effectively in individual crops. Historic datasets have been available to improve understanding of disease control requirements and to examine the consequences of different courses of action. Potential users were particularly interested to have guidance on sub-optimal use when an optimal timing had been missed because of adverse weather or other factors. Some economies in application costs can be made by combining pest and disease decisions. Greater benefits should be achieved where spray timing is optimised and unnecessary treatments are omitted. The development of DSS has produced new understanding of research on epidemiology and disease management. It is implicit that the DSS must provide clear benefits to users so that support is forthcoming to keep it up to date. In future, there is potential to broaden the scope of the PASSWORD DSS to include other aspects of oilseed crop production and to develop mechanistic models of pest and disease development so that the predictions can be updated continuously by weather data.

Acknowledgments

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PATHOLOGISTS SECTION

Plant Diseases, distribution, production and control measures

Integrated Protection in Oilseed Crops
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Comparing fungal diseases on oilseed rape in England, France and Poland

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Abstract: An analysis of data for diseases on untreated oilseed rape plants, predominantly those grown as part of the IMASCORE project in England, France and Poland during the seasons 1997/1998, to 1999/2000, has been used to demonstrate potential changes in disease occurrence due to future climate change. Many climate models predict a slight warming in North-West Europe, with milder winters and warmer summers, but the greatest impact on diseases of oilseed rape is likely to be due to changes in rainfall with increased winter rain but summer droughts. The potential effects of these changes on the timing and severity of various diseases is discussed.

Key words: climate change, powdery mildew, light leaf spot, phoma stem canker, alternaria pod spot, sclerotinia stem rot

Introduction

Many recent reports have considered the biological effects of recent changes to the climate. In north west Europe, there has been a gradual warming over the last few decades, causing effects such as plants flowering several days earlier, frogs spawning earlier and insects or birds extending their range northwards. Meteorologists have created numerous models to predict the likely future climate in different regions of the world. Most models predict that in the next 20-50 years, much of Europe will have milder and in some cases much wetter winters, and warmer, drier summers. The UK met office Hadley Centre computer models for example include coupled atmosphere-ocean general circulation models (HadCM2 and HadCM3) [www.met-office.gov.uk/research/hadleycentre/]. These are based on greenhouse emmissions increasing according to economic growth without any measures for their reduction.

The future effects of climate change on diseases of oilseed rape cannot be predicted with any certainty. It is possible, however, to compare current disease incidence and severity in areas with contrasting climates. In this paper, the occurrence of disease on untreated oilseed rape along with the local weather conditions in England, France and Poland are compared. Differences between sites, and seasons with unusually mild and wet winters or warm or dry summers may explain what is likely to become the normal situation in the future. The potential outcomes may have considerable implications for plant breeding and chemical control of diseases.

Material and methods

Disease assessments and weather data

Plots of winter oilseed rape, cv. Lipton, surrounded by guard rows, were established according to local commercial practice as part of larger experiments located at Rothamsted,

England and Cerekwica, near Poznań, Poland. Three or four replicate plots were used, the experiment design varying slightly with location and year. The incidence and severity of different diseases were recorded periodically on the leaves, stems and pods of at least ten plants per plot. Weather data were recorded locally.

L. maculans, spore trapping and disease

The occurrence of ascospores of *Leptosphaeria maculans* or *L. biglobosa*, which cause phoma stem canker and upper stem lesions, was monitored by air sampling using Burkard or Hirst-type spore samplers in locations in England, France and Poland. Trapped spores were identified and counted by microscopy and the pattern of spore release compared with meteorological data recorded locally and the onset of disease (phoma leaf spotting).

Results and discussion

Disease assessments and weather data

Rothamsted had milder winters than Poznań, with temperatures on average 5°C warmer, but summer temperatures were several degrees warmer in Poznań than at Rothamsted (Fig 1a). Rainfall was more variable at Rothamsted and was generally slightly greater than at Poznań (Fig. 1b). In both locations, the rainfall, although variable each month, was fairly evenly distributed throughout the year. Climate predictions are for milder winters but with summer temperatures up to several degrees warmer than at present. So Rothamsted may experience summer temperatures similar to those that Poznań currently experiences. Rainfall in winter is predicted to be similar to now, or higher in the far NW (Scotland and north England), but significantly lower in the summer.

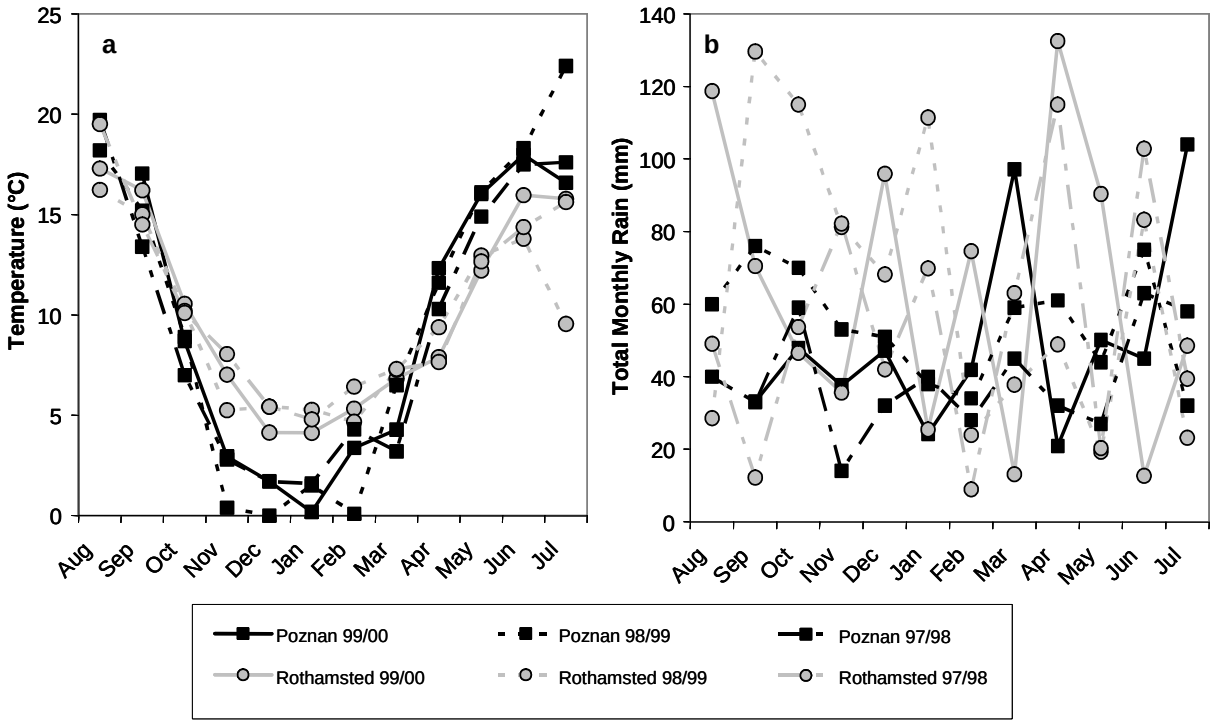

Figure 1. Mean monthly temperatures (a) and total monthly rainfall (b) at Poznan and Rothamsted, 1997-2000.

Disease assessments showed that light leaf spot (*Pyrenopeziza brassicae*) was not detected on cv. Lipton in Poznan in 1998 and 1999. Even in 2000, it was only present at very low severities on leaves and stems. Light leaf spot was not detected on pods at all in Poznan, while in England the disease progressed from leaves to stems and finally pods in all three seasons (Fig. 2). Light leaf spot in England and Poland may therefore become more severe on leaves over winter (due to wetter weather), but the low level of light leaf spot in Poznan in late spring (no disease on pods), which appears to be due to the drier and warmer conditions in late-spring and summer, might allow the future Rothamsted crop to escape serious pod damage by this disease, unless infection of the meristem is established in the spring.

Downy mildew (*Peronospora parasitica*) was present on leaves only in Poznan, reaching severities of 8, 30 and 10% in the seasons harvested in 1998, 1999 and 2000 respectively. At Rothamsted, this disease was slightly less severe on leaves generally and was absent in 1999, but it was observed at very low severities (<1%) on pods in 2000 (data not illustrated). As downy mildew is favoured by wet and humid weather, it may in future become more prevalent in the late autumn and winter.

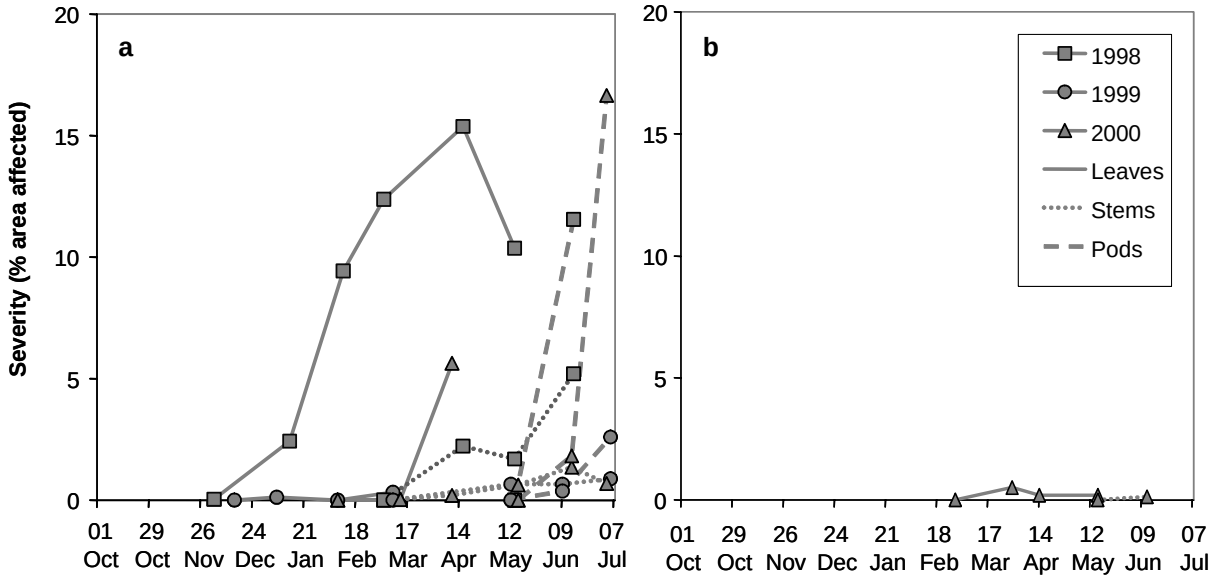

Figure 2. Mean severity of light leaf spot (*Pyrenopeziza brassicae*) on leaves, stems and pods at Rothamsted (a) and Poznan (b), 1997-2000.

Powdery mildew (*Erysiphe cruciferarum*) was often not present but occasionally became severe in a very short period. In Poznan, powdery mildew on stems increased from 0 to >50% severity (% area affected) from mid-May to mid-June 2000, but was absent in 1998 and 1999. At Rothamsted, powdery mildew on pods reached 10 and 13% incidence (% pods affected) in mid-June to early July in 1999 and 2000, respectively, but was absent in 1998 (data not illustrated). In future, severe pod infections of powdery mildew may be combined with very hot and dry conditions near to harvest, particularly in central and southern areas of Europe growing oilseed rape. This could increase the impact of this disease on heat and water-stressed plants, leading to early senescence and reduced yields.

Alternaria leaf and pod spot (*Alternaria brassicae*) was nearly always present on leaves, although often at low levels and it was always present on pods, particularly towards harvest. In Poznan, on pods it reached 16, 26 and 7 % incidence in mid-June or early July in 1998, 1999 and 2000, respectively. At Rothamsted, it reached 13, 3 and 5.5% incidence on pods in mid-June or early July of the same years. As with powdery mildew, pod infections, if combined with

increased summer temperatures may exacerbate the current yield loss due to this disease although incidence may decrease due to fewer wetness-induced infection events.

Sclerotinia stem rot (*Sclerotinia scletotiorum*) was generally rare, reaching 0.04%, 0.27% and 0% incidence in Poznan and 0%, 0% and 0.05% incidence at Rothamsted in 1998, 1999 and 2000, respectively. Previous studies suggest that sclerotinia stem rot is a problem only once in several years. It is therefore difficult to predict the effects of climate change on this disease. The impact of sclerotinia is likely to increase if the timing of flowering, and particularly petal-fall, coincides with spore release.

L. maculans, spore trapping and disease

Phoma leaf spot and phoma stem canker or upper stem lesions were very prevalent in England, France and Poland during the three seasons studied. However, the basal stem cankers were rare in Poznan, while upper stem lesions tended to be more prevalent and severe, compared to sites in France or at Rothamsted. This is thought to be because infections caused by early (autumn) spore releases in Poland often do not reach the stem due to frost causing leaves to drop prematurely, while the cold winter weather largely prevents further spore release leading to a second peak of spores released in the spring, which lead to infection of upper stems (West *et al.* 2002).

Table 1: *L. maculans* spore release and disease incidence in France, Rothamsted and Poznan

Site	1st spores trapped	No. rain days (>0.2 mm) since 1 Aug to 1st spores	1st leaf spots observed
Nancy, France	9/10/97	-	10/10/97
Nancy, France	13/9/98	**18**	
Surgères, France	21/10/97	-	27/10/97
Surgères, France	6/10/98	**19**	
St Pathus, France	25/8/97	-	17/10/97
St Pathus, France	11/9/98	**20**	
Dijon, France	12/9/98	**16**	
St Florent, France	9/9/97	**5**	25/10/97
St Florent, France	24/9/98	**20**	<9/11/98
St Florent, France	26/8/99	**11**	<9/11/99
Rothamsted, UK	8/10/97	**22**	22/10/97
Rothamsted, UK	27/9/98	**21**	22/10/98
Rothamsted, UK	14/9/99	**15**	28/10/99
Poznan, Poland	<28/9/98	**23**	9/11/98
Poznan, Poland	25/10/99	**32**	28/2/2000

With a few exceptions, spores were released approximately 20 rain days (days with over 0.2 mm of rain) after the 1st August (Table 1). However some major exceptions were in 1997 at St Florent, when spores were released after only 5 rain days since 1st August that year and 1999 at Poznan, where spores were released after 32 rain days. The model developed by Salam *et al.* (2003) predicts ascospore release following rain after 43 days suitable for maturation, as defined by temperature and rainfall parameters, since harvest. This model was not found to be completely consistent for European conditions but it remains clear that the moistness of stubble related to rain events is important in the maturation of ascospores of *L. maculans*. Leaf spotting was usually observed one to a few weeks after the first spores were released. An exception was in Poznan in 1999/2000, when spore release was relatively

late and was followed by a period of cold weather so that leaf leasions were not recorded until about 4 months later when snow had melted.

With a tendency for longer drier summers, it is probable that *L. maculans* ascospore release will be delayed. While Sun *et al.* (2001) suggest that later infections lead to less damaging cankers, in the future, crop emergence may also be delayed by drought and so spores would still affect relatively young plants. Indeed, there may be a greater synchrony of spore release with autumn rain as there is currently in Australia, resulting in spore release being concentrated into a shorter time period. As a result, there could be a greater need for resistant cultivars and even well-timed fungicide applications.

Acknowledgements

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The effect of sulphur, magnesium and boron fertilisation of the spring rape on the occurrence of diseases on plants and fungi composition on harvested seeds

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Abstract: The effect of sulphur, magnesium and boron and fungi composition on harvested seeds on spring oilseed rape infections by leaf and seed pathogens was tested in central Poland over a three-year period. Powdery mildew (*Erysiphe cruciferarum*) appeared at high intensity over the whole plants during two years, while downy mildew (*Peronospora parasitica*) on leaves and dark leaf and pod spot (*Alternaria* spp.) occurred moderately. Black leg (*Phoma lingam)* and sclerotinia stem rot (*Sclerotinia sclerotiorum)* were observed only sporadically. There was no apparent effect of sulphur alone on the health of tested plants. Significant improvements in plant health were obtained when sulphur and fungicides were combined. Among pathogenic fungi isolated from seeds *Alternaria brassicae* was the most frequent, followed by *Alternaria alternata* while other species were noted only sporadically. There was no effect of sulphur on seed infection rates.

Key words: spring rape, sulphur, magnesium, boron, fertilisation, diseases, fungi, seeds, fungicides

Introduction

Winter oilseed rape is preferred in Poland, however, in some years weather conditions press for cultivation of its spring form. Shorter growing season frequently results in a lower yield of spring oilseed rape, which may be further reduced if weather conditions are inadequate.

Rape requires sufficient sulphur content in the soil. Sulphur influences the yield of seeds and oil nitrogen uptake as well as composition of fatty acid and glucosinolate. An overall sufficient level of sulphur promotes healthy plants. Reductions of disease occurrence under increased soil sulphur content were presented by many authors (Schnug and Ceynowa 1990, Schnug & Haneklaus 1995, Schnug et al. 1995, Haneklaus at al.1999, Sadowski et al. 2000, 2002, 2003, Drozdowska et al. 2002).

In the early 1950s sulphur in most Polish soils was abundant. However during the last two decades emission of sulphur into the atmosphere and subsequently, its soil content has been profoundly reduced. Grzebisz and Fotyma (1996) showed serious lack of sulphur in northwest Poland's soils almost a decade ago.

Rape also demonstrates a high boron and magnesium requirement, a lack of these elements may negatively influence plant growth and development, induces flower necrosis, reduces the number of pods and seeds and results in a higher susceptibility to diseases as well as an overall reduction in seed yield (Grzebisz and Gaj 2000).

Since it is anticipated that atmospheric emission of sulphur in Poland will further diminish, the occurrence of numerous oilseed rape diseases may increase. The main objective of this research was to evaluate the effect of supplemental fertilization of rape with sulphur. Additionally, the supplementary benefit of applied boron and magnesium were tested to evaluate their influence on disease incidence.

Material and methods

The field study was conducted during, 2001, 2002, and 2003 at Research Centre for Cultivars Testing in Chrząstowo near the city of Bydgoszcz on the good wheat soil with pH 6.4. Soil was fertilized in spring with 140 kg ha^{-1} N, 80 kg ha^{-1} P, and 90 kg ha^{-1} K. Sulphur content was about 0,7 mg SO_4 in 100 g of soil. The treatments included:

+ untreated control
+ soil applied sulphur at 40 kg ha^{-1}
+ foliage applied sulphur at 40 kg ha^{-1} (20 kg rosette stage + 20 kg stem extension phase)
+ soil applied sulphur at 40 kg ha^{-1} + boron at 0,5 kg ha^{-1}
+ soil applied sulphur at 40 kg ha^{-1} + magnesium at 1 kg ha^{-1}
+ soil applied sulphur at 40 kg ha^{-1} + fungicide treatment 1 + fungicide treatment 2

Fungicide 1 was Caramba 60 SL (metconazol), fungicide 2 was Konker 415 S.C. (winchlozolin +carbendazim). Sulphur was applied in the ionic form as sodium sulphate. Soil application was prior to sowing. Foliage application was at the rosette phase and was repeated at the stem extension phase. Magnesium was applied as magnesium sulphate and boron was applied as di-sodium tetra borate, both as foliar application. Plot size was 16,5m^2. Spring rape cv. Margo was sawn around mid April. The experimental design was randomised split plot with four replications. All data were separated by LSD at P=0.05.

Visual estimates of disease infections were made once or twice during the growing season and for *Peronospora parasitica* and *Erysiphe cruciferarum* were based on the scale 0-5 (Sadowski 1987, Penaud 1999). For *Alternaria* spp. were used scale 0-4, and on leaves according Evens & Gladders (1981), on pods Babadoost & Gabrielson (1979). Mycological analysis of seeds was carried out by plating 100 seeds from each replication on PDA medium. Additionally, the filter paper method (Capelli *et. al.* 1998) was used. Under this procedure seeds were surface disinfected with NaOCl and placed on filter paper soaked with 2,4-D to inhibit germination.

Results and discussion

Overall the occurrence of powdery mildew was low in 2001 but in 2002 occurred at higher intensity. When averaged over a 3 years period, sulphur application reduced intensity of this disease. The maximum effect was obtained in combination where sulphur and fungicides were applied (Table 1). Downy mildew occurred every year with low intensity and no differences between combinations (Table 2). Dark leaf was observed at higher intensity only in 2002 but its intensity was not influenced by sulphur. Significant reduction of disease development was observed after application of fungicides (Table 3). Black spot on pods was observed every year, with higher intensity in 2001. Fungicide treatments reduced black spot occurrence on pods but effect of sulphur, boron and magnesium was not significant (table 4). Because of the occurrence of black spot on pods, mycological analysis of seeds for occurrence of *Alternaria* spp. was conducted. Seed analysis showed that higher intensity of symptoms of black spot was correlated with the higher occurrence of A*lternaria brassicae.* Apart from fungicide sprays other experimental factors had no impact on the number of seeds infected with *Alternaria brassicae.* (Table 5). In each year a high occurrence of *Alternaria alternata* was observed on seeds. (Table 6). Seeds infected with *Alternaria alternata* were often settled by *Gonatobotrys simplex* (data not presented). Mean yield of seeds averaged over 3 years did not depend upon applied sulphur with the highest yield obtained in combinations where fungicides were used.

Tab. 1. Occurrence of powdery mildew (*Erysiphe cruciferarum*)

Combination	DI (in%)			
	2001	**2002**	**2003**	**2001-2003**
So - control	6,8 a*	83,3 a	34,0 a	**41,4 a**
S - 40 kg soil	5,5 ab	80,0 a	33,0 a	**39,5 bc**
S – 40 kg foliar	5,0 ab	80,7 a	34,0 a	**39,9 b**
S – 40 kg soil + B	3,3 ab	76,7 a	34,0 a	**38,0 d**
S - 40 kg soil + Mg	3,1 b	81,3 a	34,0 a	**39,5 bc**
S - 40 kg soil + fungicides	2,0 b	78.0 a	3,6 b	**27,9 e**

**/ Values in the same column followed by different letters are significantly different*

Tab. 2.Occurrence of downy mildew (*Peronospora parasitica*)

Combination	DI (in%)					
	I term			II term		
	19.06. 2001	19.06. 2002	**2001-2002**	27.06. 2001	10.07. 2002	**2001-2002**
So - control	3,1	5,5	**4,3**	7,4 a	5,6 a	**6,5**
S - 40 kg soil	2,3	6,8	**4,6**	8,1 a	5,1 a	**6,6**
S – 40 kg foliar	2,8	6,9	**4,8**	8,5 a	5,6 a	**7,0**
S – 40 kg soil + B	2,9	6,7	**4,8**	9,3 a	5,5 a	**7,4**
S - 40 kg soil + Mg	2,2	5,5	**3,8**	7,1 a	5,3 a	**6,2**
S - 40 kg soil + fungicides	2,4	6,8	**4,6**	8,5 a	3,6 a	**6,0**

Tab. 3. Occurrence of dark leaf *(Alternaria* spp) on leaves

Combination	19.06.2002		10.07.2002	
	%	**DI**	**%**	**DI**
So - control	6,8	1,7	26,8 a	6,9 a
S - 40 kg soil	5,5	1,4	31,3 a	8,0 a
S – 40 kg foliar	4,3	1,1	29,8 a	7,7 a
S – 40 kg soil + B	3,8	0,9	24,5 a	6,2 a
S - 40 kg soil + Mg	5,3	1,5	29,5 a	7,4 a
S - 40 kg soil + fungicides	4,5	1,1	7,5 b	1,9 b

Results of this study showed inconsistent effects of sulphur on disease occurrence. Similar irregularities were previously reported by Sadowski et al. 2000, and 2003, who found a decrease of dark leaf and moderate decrease of black spot on the pods as well as no differences between soil or foliar application of sulphur. In another study conducted in the same year application of sulphur resulted in lower intensity of black spot on pods but not on leaves (Sadowski at al.2002). Supplementary benefit of magnesium and boron applied in this study was rather minor. Magnesium reduced the occurrence of *Alternaria* on leaves and stems at earlier sowing date with no impact at later sowing date. Boron decreased occurrence of dark leaf spot only once in one year (Sadowski at al. 2002). As previously reported by Sadowski et al.2002, and 2003, application of sulphur had no influence on intensity of downy mildew.

Tab. 5. Occurrence of *Alternaria brassicae* on seeds

Combination	% of infected seeds			
	2001	**2002**	**2003**	**2001-2003**
So - control	16,2	2,5 ab	0,2	**6,3 a**
S - 40 kg soil	13,8	2.8 ab	0,2	**5,6 a**
S – 40 kg foliar	15,2	2,2 ab	0,0	**5,8 a**
S – 40 kg soil + B	15,2	3,0 ab	0,0	**5,8 a**
S - 40 kg soil + Mg	14,2	3.0 ab	0,0	**5,7 a**
S - 40 kg soil + fungicides	9,5	0,2 b	0,0	**3,2 b**

Tab. 6. Occurrence of *Alternaria alternata* on seeds

Combination	% of infected seeds			
	2001	**2002**	**2003**	**2001-2003**
So - control	52,8	39,0	56,5	**49,4 ab**
S - 40 kg soil	68,2	33,5	51,5	**51,1 ab**
S – 40 kg foliar	65,5	42,8	50,0	**52,8 a**
S – 40 kg soil + B	63,0	30,6	56,5	**50,0 ab**
S - 40 kg soil + Mg	65,5	28,2	65,0	**52,9 a**
S - 40 kg soil + fungicides	61,8	27,2	41,0	**43,4 b**

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The contribution of cultivar resistance and fungicides to disease control in winter oilseed rape in England

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Abstract: Phoma stem canker (*Leptosphaeria maculans*) continues to cause significant yield losses in England on winter oilseed rape, despite widespread use of fungicides. This paper reviews results from a series of eight experiments that aimed to quantify the contribution of cultivar resistance and fungicides to stem canker control. Experiments were done during 1999-2002 with 3-5 cultivars per year and autumn, winter and autumn + winter fungicide treatments at ADAS Boxworth (high risk area for stem canker) and ADAS High Mowthorpe (moderate risk for stem canker and light leaf spot (*Pyrenopeziza brassicae*). Significant yield differences were recorded between cultivars in six experiments, but the highest yields were not associated consistently with the most disease resistant cultivars. Yield responses to fungicide were significant in five experiments and in each case, two sprays applied in autumn gave comparable yield responses (0.33-0.79 t/ha) to a four-spray programme applied in autumn and winter and to a winter only programme. Autumn fungicides gave better control of moderate and severe cankers than winter sprays. Fungicide timing was less critical at High Mowthorpe where stem canker was not severe and control of light leaf spot contributed to yield responses. Fungicides contributed to disease control and yield responses to a greater extent than cultivar resistance.

Key words: phoma leaf spot, stem canker, light leaf spot, yield response

Introduction

Despite widespread use of fungicides on winter oilseed rape, diseases are estimated to have caused yield losses of up to £80 million/annum in the UK (Fitt et al., 1997). Phoma stem canker (*Leptosphaeria maculans*) is regarded as a monocyclic disease with air-borne ascospores producing phoma leaf spots that initiate stem canker lesions after systemic mycelial growth within the petiole reaches the stem (West et al., 1999). Early phoma leaf spotting produces early stem cankers and these are most likely to become moderate or severe by harvest and reduce yield (Zhou et al., 1999). The development of phoma leaf spot shows marked seasonal and regional variation, which makes decision making complex and difficult to optimise. Fungicide treatments are often ineffective against phoma stem canker, because they are either not well-timed or unnecessary. Although disease resistant cultivars are available, they have not been fully exploited. This paper reviews a series of experiments that aimed to quantify the contribution of cultivar resistance and fungicides to stem canker control. A second objective was to test the hypothesis that disease control in autumn was required to control moderate and severe stem canker and for yield benefits.

Materials and methods

Cultivar x fungicide interactions were investigated in a total of eight replicated small plot (minimum plot area 40 m^2) field experiments during 1999-2002 using natural epidemics of

disease at ADAS Boxworth (high canker risk) and ADAS High Mowthorpe (moderate canker and moderate light leaf spot risk). Cvs. Pronto (canker resistance rating 5) and Apex (canker resistance rating 6) were used as standards in all experiments, whilst Alpine (resistance rating 5) and more resistant cvs Contact, Escort, Licrown and Lutin were included according to availability (canker resistance rating 7) (Table 1). Plots were drilled in late August or early September with seed rates to achieve target plant populations of 40 plants/m^2 for the hybrid cultivar Pronto and 80-100 plants/m^2 for other cultivars.

The crops were grown with fertiliser and agronomic treatments (except fungicides) according to local farm practice. In 2002, all treatments were evaluated with and without an additional 50 kg/ha of nitrogen fertiliser at sowing, but the nitrogen effects were small and the results presented in this paper have been averaged across nitrogen treatments. Fungicide regimes based on flusilazole + carbendazim (100 + 50 g a.i./ha as Punch C) were applied by Oxford Precision Sprayer in 200-225 litres water/ha in autumn/early winter or winter/spring as two-spray programmes or on all dates (Table 1). Disease assessments were carried out from disease onset in autumn at 4-6 week intervals up to harvest and recorded the incidence and severity of leaf (% leaf area), stem (0-4 index, plot means on 0-100 scale) and pod (% pod area) diseases (Gladders & Symonds, 1995). The final stem canker assessments were made at the seed green to green brown mottled stage. The plots were combine harvested after desiccation at Boxworth or swathing at High Mowthorpe and yields were adjusted to 90% dry matter.

Results and discussion

There were rapid increases in the incidence of phoma leaf spot symptoms each year and epidemic onset (>20% plant affected) varied between 13 October and 14 November at Boxworth (Fig. 1) and 12 October and 9 December at High Mowthorpe. Plants had only reached the 3-4 leaf stage in six of the eight experiments when phoma first developed, but plants were very large (GS 1,12) at Boxworth in 2001 (Table 1). The stem canker index was consistently higher at Boxworth than at High Mowthorpe. This reflected the higher incidence and severity of phoma leaf spotting during the autumn and winter at Boxworth. There were significant differences in canker indices between cultivars in all four years at Boxworth and in 2001 at High Mowthorpe (Table 2). Pronto had a higher stem canker index than Apex at Boxworth, but the severity indices on Apex did not differ significantly from more resistant cultivars except when Apex had a lower severity index than Licrown in 2000.

Fungicides gave significant reductions in stem canker indices in six experiments, but no control was achieved in 1999 and 2001 at High Mowthorpe. There were significant differences between the two spray programmes and between both two-spray programmes and the full programme at Boxworth. At Boxworth, autumn treatments were more effective than winter treatments in 2001and 2002, but winter treatments were better than autumn sprays in 1999 and 2000. At High Mowthorpe, fungicides gave control of stem canker in 2000 and 2001, but there were no significant differences between the fungicide treatments.

There were significant differences in yield between cultivars in all six experiments carried out during 2000-2002. Canker-resistant cultivars Licrown and Escort produced higher yields than more susceptible cultivars at High Mowthorpe in 2000 and 2002, but the converse was true at Boxworth. There were significant positive yield responses to all fungicide treatments at Boxworth in 2002 and High Mowthorpe in 2001 and 2002. Yield increases also occurred with an autumn programme at Boxworth in 2001 and autumn and full treatments at High Mowthorpe in 2000. Light leaf spot developed in experiments at High Mowthorpe, affecting 3-4% leaf and stem area in untreated plots, but was well controlled by all the fungicide treatments. There were no yield responses at either site in 1999.

Table 1. Cultivars, date of sowing, growth stage and date at onset of phoma leaf spotting in experiments at Boxworth and High Mowthorpe in harvest years 1999-2002.

Site	Boxworth				High Mowthorpe			
Year	1999	2000	2001	2002	1999	2000	2001	2002
Date of sowing	7 Sep	31 Aug	1 Sep	30 Aug	7 Sep	27 Aug	30 Aug	31 Aug
Cultivars	Pronto Apex Licrown Alpine	Pronto Apex Licrown Alpine Contact	Pronto Apex Escort Lutin	Pronto Apex Escort	Pronto Apex Licrown Alpine	Pronto Apex Licrown Alpine Contact	Pronto Apex Escort Lutin	Pronto Apex Escort
Dates of fungicide applications								
Autumn 1	20 Oct	27 Oct	14 Nov	17 Oct	6 Nov	9 Nov	14 Nov	1 Nov
Autumn 2	1 Dec	29 Nov	13 Dec	30 Nov	9 Dec	17 Jan	5 Jan	10 Dec
Winter 1	21 Jan	17 Jan	17 Jan	9 Jan	18 Feb	22 Feb	9 Feb	8 Feb
Winter 2	9 Mar	22 Feb	5 Mar	15 Mar	21 Apr	31 Mar	14 Mar	19 Mar
Epidemic onset	20 Oct	27 Oct	14 Nov	13 Oct	9 Dec	26 Oct	12 Oct	1 Nov
GS at phoma onset	1,07	1,03	1,12	1,04	1,04	1,04	1,04	1,07

The most severe canker developed at Boxworth in 2002 and examination of the effects of fungicides on individual cultivars showed that autumn fungicide treatments were more effective in controlling moderate and severe cankers than winter treatments. The full fungicide programme gave the highest incidence of healthy stems (Fig. 2) but yields of autumn and full programmes were not significantly different. Cultivar resistance provided only partial control of stem canker as untreated canker indices on cvs Pronto, Apex and Escort were 73, 55 and 58 respectively in 2002. Autumn fungicide treatments gave indices of 40, 25 and 21 on cvs Pronto, Apex and Escort demonstrating that good disease resistance combined with fungicides provided the lowest disease severity.

These experiments demonstrated the variability of phoma development at sites in eastern and northern England and their subsequent impact on canker development, fungicidal control and yield response. There were benefits from using cultivars with good resistance to stem canker at some sites, though factors other than disease influence the yield performance of cultivars.

Fungicides gave significant yield responses in five experiments and these were obtained with two spray programmes. Overall, there were small differences between autumn and winter programmes, but mean responses of 0.3 t/ha (Table 3) would be improved if treatments were not made to non-responsive sites. At Boxworth in 2001 and 2002, there were indications that yield benefits of about 0.2 t/ha might be obtained by applying fungicide sprays in autumn rather than in winter. This was associated with better control of moderate and severe stem cankers that cause premature ripening and reduce yield (West et al., 1999; Zhou et al., 1999). Winter sprays provided control of slight canker lesions, but these had little effect on yield. The cost of a single fungicide treatment equates to about 0.1 t/ha of yield and an additional yield response of 0.2 t/ha would provide a benefit worth 50 euro/ha at current crop values. At High Mowthorpe, all the fungicide programmes gave good control (>90%) of light leaf spot

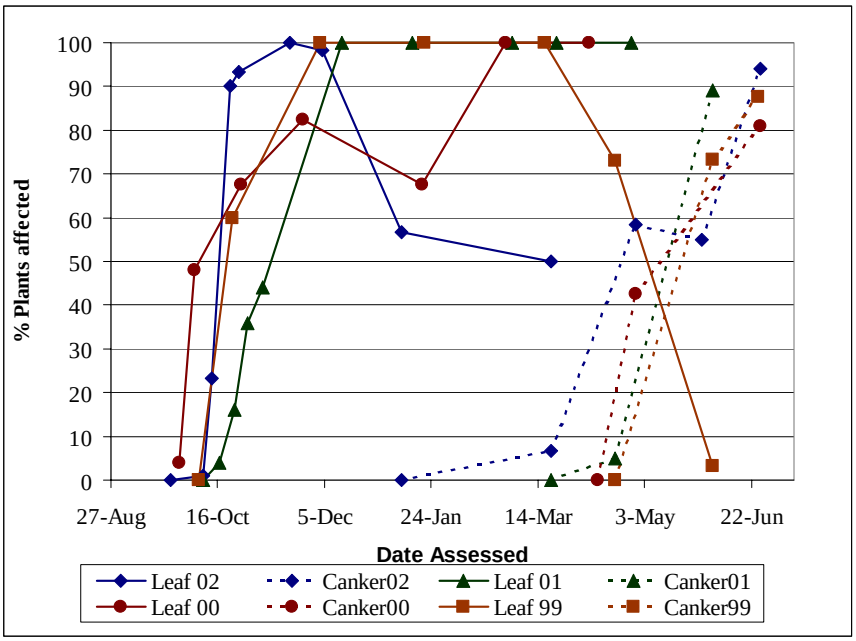

Figure 1. Development of phoma leaf spot and stem canker on cv. Apex at ADAS Boxworth 1999-2002.

Table 2. Yield in relation to cultivar and fungicide treatments at Boxworth and High Mowthorpe in harvest years 1999-2002.

	Yield (t/ha) at 90% dry matter								
Site	Boxworth				High Mowthorpe				
Year	1999	2000	2001	2002	1999	2000	2001	2002	Mean
Variety									
Pronto	2.81	3.95	3.48	3.99	2.47	3.77	3.38	3.28	3.39
Apex	2.88	4.09	3.28	3.88	1.98	3.63	3.14	3.28	3.27
Alpine	2.63	3.82			2.25	3.73			3.11
Contact		3.76				4.32			4.04
Escort			3.45	3.60			2.60	3.83	3.37
Licrown	2.97	3.77			2.84	4.07			3.41
Lutin			3.01				3.23		3.12
SED	0.128	0.060	0.099	0.078	0.132	0.154	0.145	0.085	
df	30	57	45	58	45	56	45	58	
F pr.	0.075	<0.001	<0.001	<0.001	0.787	<0.001	<0.001	<0.001	
Fungicide									
Nil	2.73	3.83	3.14	3.28	2.40	3.58	2.56	3.16	3.085
Autumn	2.67	3.85	3.47	4.02	2.31	4.04	3.35	3.58	3.41
Winter	2.92	3.88	3.29	3.80	2.44	3.86	3.11	3.62	3.365
Full	2.96	3.95	3.32	4.19	2.40	4.12	3.32	3.49	3.47
SED	0.128	0.053	0.099	0.096	0.132	0.138	0.145	0.098	
df	30	57	45	58	45	56	45	58	
CV%	12.8	4.4	8.4	7.1	15.6	11.2	13.3	8.5	
F pr.	0.076	0.128	0.018	<0.001	0.787	0.001	<0.001	<0.001	
Interaction F pr.	0.118	0.636	0.384	0.919	0.079	0.474	0.331	0.187	

and this contributed to the rather higher yield responses at High Mowthorpe compared with Boxworth, despite lower canker indices.

Table 3. Phoma stem canker indices pre-harvest in relation to cultivar and fungicide treatments at Boxworth and High Mowthorpe in harvest years 1999-2002.

	Phoma stem canker index (0-100 scale) pre-harvest								
Site	Boxworth				High Mowthorpe				
Year	1999	2000	2001	2002	1999	2000	2001*	2002	Mean
Variety							(angles)		
Pronto	31.5	23.8	24.4	46.9	15.0	0.3	10.5	1.3	19.18
Apex	25.9	15.0	16.2	31.8	17.3	1.8	3.3	0.5	13.96
Alpine	32.9	19.9			9.2	1.8			15.95
Contact		12.6				1.3			6.92
Escort			13.1	35.1			7.4	0.8	14.11
Licrown	23.2	23.0			20.2	1.6			17.00
Lutin			15.8				2.9		9.34
SED	3.70	2.629	1.746	3.27	4.22	0.92	2.04	0.54	
df	27	57	45	58	45	57	45	58	
CV%	31.9	39.4	28.4	29.8	77.4	191.0	48.8	215.6	
F pr	0.043	<0.001	<0.001	<0.001	0.078	0.448[1]	<0.001[1]	0.265[1]	
Fungicide									
Nil	42.7	34.6	29.9	61.9	19.2	4.1	12.8	1.3	25.80
Autumn	33.1	23.0	12.3	28.6	15.9	0.2	3.9	0.5	14.69
Winter	24.7	14.1	20.2	47.2	15.8	0.9	4.6	1.3	16.10
Full	13.1	3.7	7.2	14.0	10.8	0.3	2.8	0.4	6.52
SED	3.70	2.35	1.75	3.77	4.22	0.8	2.04	0.62	
df	27	57	45	58	45	57	45	58	
CV%	31.9	39.4	28.4	29.8	77.4	191.0	48.8	215.6	
F pr	<0.001	<0.001	<0.001	<0.001	0.268	<0.001	<0.001	0.311	
Interaction F pr	0.827	<0.001	0.041	0.375	0.675	0.615	0.378	0.405	

1 = data had a skewed distribution. * = Angular transformed data presented.

The effect of autumn, winter and full fungicides on final stem canker indices and yield related well to the timing and duration of the phoma leaf spot epidemic. In 2002, phoma leaf spotting was most apparent in late autumn and this resulted in early canker development in spring and the most severe canker lesions by harvest. Autumn sprays were more effective than winter sprays in this situation. The later phoma leaf spot epidemic in 2000 resulted in moderate canker severity by harvest and better control of canker from winter fungicide treatments. Cultivar x fungicide interactions were significant only for canker indices at Boxworth in 2000 and 2001 (Table 2).

The relationships between phoma leaf spot and canker and between canker severity and yield are being investigated in the PASSWORD Decision Support System (Gladders et al., this volume). This will provide new guidance on both disease development and yield loss so that fungicides can be appropriately targeted.

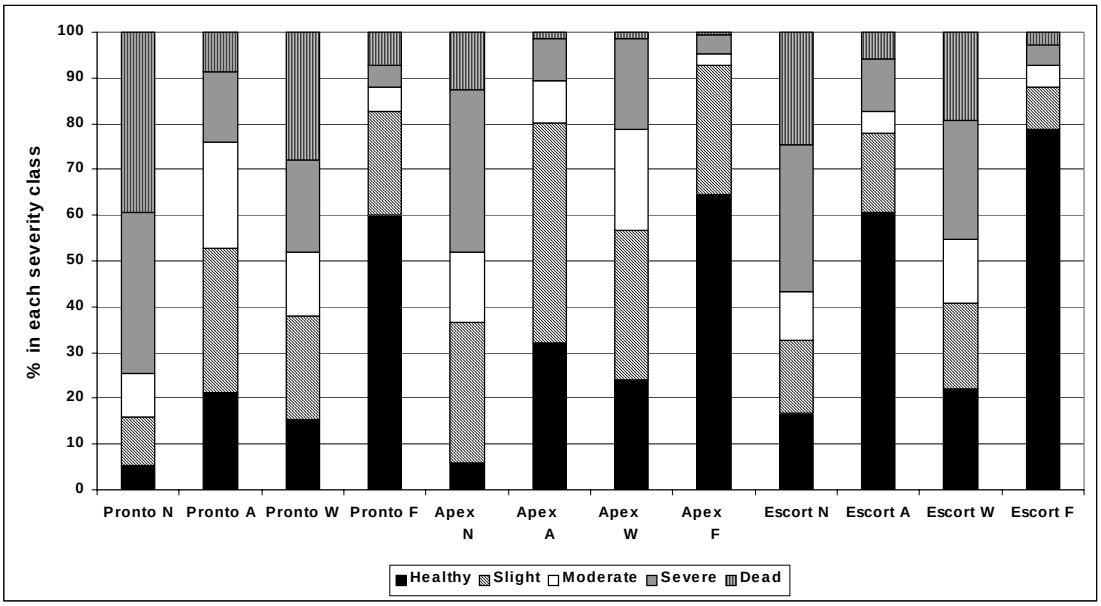

Figure 2: Incidence of healthy stems and stem canker by severity class in relation to cultivar and fungicide treatments (N – No fungicide, A – 2 autumn fungicides, W – 2 winter fungicides, F – 2 autumn + 2 winter fungicides)

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Sclerotinia stem rot

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Disease/yield loss analysis for *Sclerotinia* stem rot in winter oilseed rape

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Abstract: *Sclerotinia* stem rot is the most important fungal pathogen in winter oilseed rape in Germany. Preventive fungicide treatments are routinely applied at flowering stage to control this disease. The data evaluation of field trials conducted by German state extension services between 1991-2003 demonstrate that fungicide treatments during flowering result in an average additional yield of 2.41 dt/ha. However, an additional yield of 3.32 dt/ha is necessary to cover the costs of a fungicide treatment. This discrepancy leads to about 67 % uneconomic fungicide treatments against *Sclerotinia sclerotiorum*. In three different field trials the influence of infection parameters (inoculation date, level of infection), cropping practices (sowing time, seed density) and the compensation potential of a hybrid (Talent) and a line cultivar (Prince) were examined. An inoculation at full bloom results in higher yield losses than an inoculation at the end of flowering. For sowing time and seed density, no clear results were obtained in the two experimental years 2002 and 2003. The compensation potential seems to be weather dependent. Increasing plant reduction leads to increasing thousand seed weight.

Key words: oilseed rape, *Sclerotinia sclerotiorum*, damage threshold, disease/yield loss relationship, cost-effectiveness

Introduction

The economically most important pathogenic fungus in German winter oilseed rape production is *Sclerotinia sclerotiorum*. Yield losses of up to 50 % due to *Sclerotinia* stem rot have been reported (POPE et al., 1989, LANDSCHREIBER, 2004). The increase in rapeseed growing areas in Germany, up to 1.3 million ha in recent years, have caused a spread of this disease especially in areas with a high rapeseed production.

The appearance of *Sclerotinia* stem rot is predominantly dependent on weather conditions and thus disease incidence differs greatly in different years. For *S. sclerotiorum* infection, temperatures above 10°C, a relative humidity above 90 % for at least 36 h, sporulation of apothecia and petal fall (AHLERS and HINDORF, 1987) are required. The first symptoms of a *Sclerotinia* infection are not visible until the end of flowering, when a fungicide treatment has already taken place. Thus, preventive fungicide applications are frequently applied in practical agriculture. Investigations dealing with the economic efficiency of fungicide treatments in rapeseed show that a high percentage of fungicide treatments are inefficient (KRÜGER and STOLTENBERG, 1983, WAHMHOFF, 2000, LANDSCHREIBER, 2004). Due to uncertain estimations of the disease incidence in different years and the high potential of yield reduction by *Sclerotinia* stem rot, growers apply fungicide treatments to ensure yields.

A weather based prognosis model (SKLERO) developed by FRIESLAND (2000) in order to estimate the disease incidence of *S. sclerotiorum* predicts the probability of an infection but not the expected disease incidence or crop loss. Based on this existing model, a new decision support model is currently being developed at the University of Goettingen which will also include field specific data like crop rotation, tillage and soil type. An economic damage

threshold will be included to come to a decision whether a fungicide treatment will be cost-effective. With the help of this decision support model, inefficient fungicide treatments should be avoided.

A disease/yield loss relationship was established to develop an economic damage threshold of *S. sclerotiorum* in winter oilseed rape. In three different field trials different infection parameters (inoculation date, level of infection), cropping practices (sowing time, seed density) and the compensation potential were examined on artificially inoculated winter oilseed rape plants. The cost-effectiveness of fungicide treatments was evaluated on the basis of field trials of the German state extension services.

Material and methods

Field experiments

In the years 2002 and 2003, three different field trials were done in Goettingen, Lower Saxony in order to develop an economic damage threshold for *S. sclerotiorum* in winter oilseed rape. Natural *Sclerotinia* infections were excluded, as the field experiments were done in fields where oilseed rape had not been grown for at least ten years. Plots were arranged in a randomized block design with four replications in each experiment and a plot size of 1.25 m x 12 m (15 m²). Each field trial was conducted with a hybrid (Talent) and a line cultivar (Prince) to examine cultivar responses to *Sclerotinia* infection. Sowing was done on 21st of August 2001 and 26th of August in 2002 with a seed density of 48 seeds/m² for 'Talent' and 60 seeds/m² for 'Prince'.

Plant density was determined at growth stage (GS) 31-33 and adjusted to 50 plants/m² for 'Prince' and 40 plants/m² for 'Talent'. Insecticides and herbicides were applied as necessary. In autumn and spring, plant growth regulation was conducted with Caramba (metconazole, in autumn) and Moddus (Trinexapac-methyl, in spring).

Damage effect of S. sclerotiorum *in dependence on level and date of infection*

The inoculation was done at two different growth stages (GS 61-65 and GS 71) to investigate the effect of the date of infection. This field trial included eight different infection levels (0-70 % with steps of 10 %) for each inoculation date.

Compensation potential of winter oilseed rape

Late plant losses (0-70 %), as occurring after natural *Sclerotinia* infections, were simulated at growth stage 69-71 in order to examine the compensation potential of winter oilseed rape. Plant density was reduced by cutting the corresponding number of plants at 30-40 cm above ground.

Influence of different cropping practices on the disease/yield loss relationship

Sowing time and seed density were varied, with an early (10th to 15th August) and normal (20th to 25th August) sowing date at a low (35/45 seeds/m², 'Prince'/'Talent') and high (60/50 seeds/m², 'Prince'/'Talent') seed density. Inoculation was done at GS 67-69.

Crop loss assessment

The yield was determined for the whole plot and referred to 9 % humidity. Thousand seed weight (TSW) was measured by weighing a sub-sample of 1000 seeds from each plot.

Inoculum production and artificial field inoculation

Artificial inoculation was conducted with wooden toothpicks infected with fungal inoculum. The toothpicks were autoclaved in PDB medium and infected with agar plugs overgrown with mycelium of *S. sclerotiorum* (two different isolates originated from oilseed rape). The inoculated toothpicks were incubated at room temperature until complete spread of the fungus but without sclerotia production having started.

Disease incidence levels were adjusted in the plots in steps by 10 %, ranging from 0 – 70 %, by inoculating the corresponding number of plants per plot. One toothpick was inserted into a

leaf axil in mid plant height. *Sclerotinia* disease incidence (percentage of infested plants per plot) was assessed in the field at GS 81-83.

Economic validation

Data from official field trials of the German state extension services (Mecklenburg-Western Pomerania, Schleswig-Holstein, Lower Saxony, Brandenburg, Saxony, Thuringia, Rhineland-Palatinate, Bavaria) since 1991 were evaluated in order to analyse the economic efficiency of fungicide applications against *S. sclerotiorum*. Application costs included fungicide prices plus VAT, variable application costs and losses due to tractor passage.

The calculation of the variable application costs was based on data published by the Association for Technology and Structures in Agriculture (KTBL) and consisted of machine costs (standard tractor with all-wheel drive, 75-92 kW, mounted plant protection equipment, 1000 l, 15 m working width) and labour costs (labour time requirement: 200 l per hectare application volume, field size 2 ha, foreman wage costs). Yield losses due to tractor passage during a fungicide treatment at time of full bloom are estimated at 2.6 % per ha. Oilseed rape producer prices are average values over one growing season (Table 1)

Table 1: German oilseed rape producer prices (ZMP) and variable application costs for the period of investigation

Year	Oilseed rape producer price [EUR/t]	Variable application costs [EUR/ha]
1991	349.47	6.96
1992	155.41	7.50
1993	193.35	7.50
1994	196.85	8.70
1995	187.97	8.70
1996	216.32	8.64
1997	242.74	8.64
1998	195.01	7.96
1999	161.26	7.96
2000	189.67	9.17
2001	221.77	9.17
2002	240.14	7.45
2003	228.71	7.45

(Data source: Agricultural market information agency, ZMP, Bonn)

Statistical analysis

Linear regression analysis was used to quantify the relationship between *S. sclerotiorum* incidence and yield and thousand seed weight. Statistical parameters were calculated using the Statgraphics Plus System (version 5.1).

Results and discussion

Field trials

The field trial on the affect of time of infection on the crop loss indicated that in 2002 the disease incidence, resulting from the artificial inoculation, was at the same level at both inoculation times

whereas in 2003 inoculation at GS 71 resulted in higher disease incidence than at GS 61-65. This effect may be due to different weather conditions in the two experimental years. For both cultivars the disease/yield loss analysis shows that the crop loss resulting from infections at GS 61-65 are higher than at GS 71 (data not shown). The study of SHIVPURI et al. (1999) also shows higher yield losses due to *Sclerotinia* infections at earlier growth stages than later infections in mustard and rapeseed. A more progressed yield formation may be the reason for lower yield losses due to infections at late growth stages.

The field trial on the compensation potential of winter oilseed rape shows a clear yield reduction with increasing plant reduction whereas the TSW increases (Fig. 1). A reduction of plant density at the beginning of pod formation (GS 71) can not be compensated completely because the plants can not produce new branches at this late growth stage. The only possibility to compensate late plant losses is to increase the TSW.

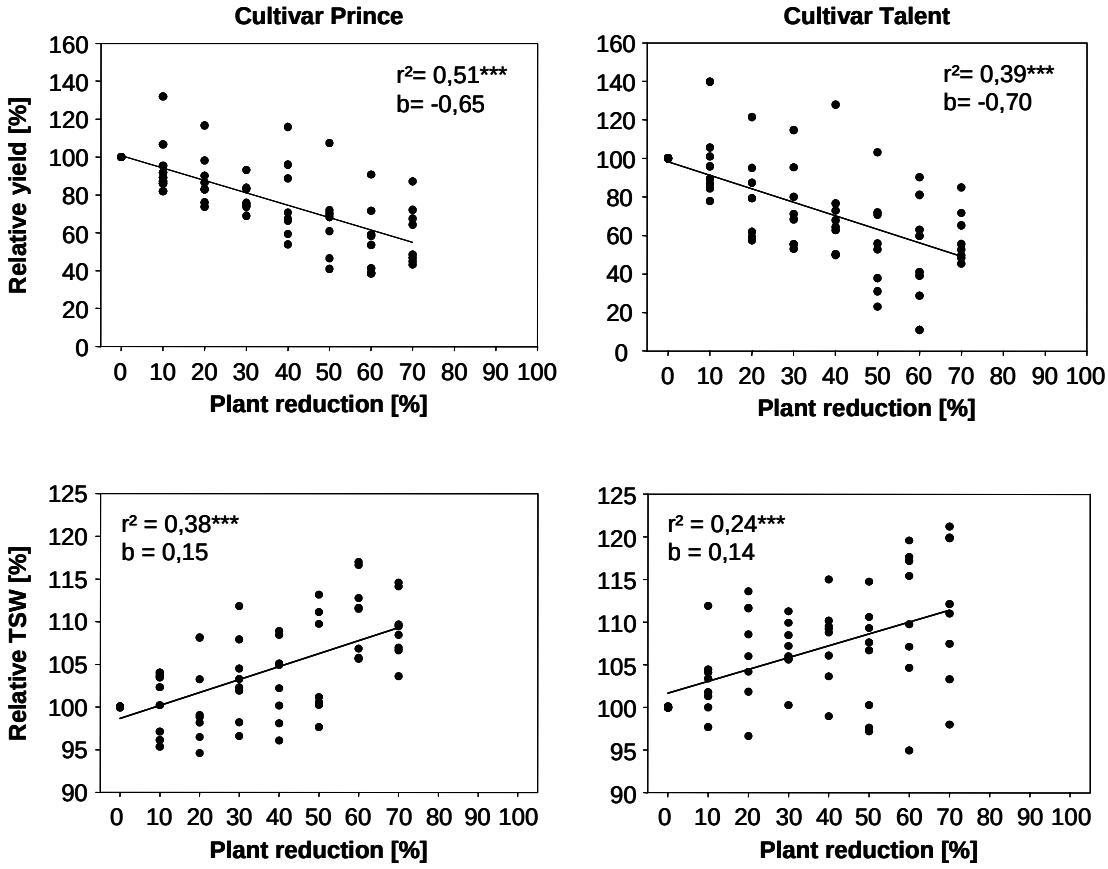

Fig. 1: Relative yield and thousand seed weight (TSW) in relation to plant reduction at GS 71, cv. Prince and cv. Talent, Goettingen, average over 2002 and 2003. Asterisks indicate significant differences according to the Durbin-Watson statistic (P = 0.01).

SCHLOTT and SAUERMANN (2003) reduced plant density by 25, 50, 75 and 100 % at different growth stages, using the same method as described above. The results of this study agree with the results of our field trials. With increasing plant reduction, the TSW increased. The compensation effect depends on the growth stage, with the strongest effect being observed at the end of flowering/beginning of seed formation. From the beginning of seed formation onwards, the compensation of plant losses decreases and approaches the percentage of reduced plants. Because the number of pods is already determined at the end of flowering, the compensation of plant losses then takes place in the form of better seed formation.

The two-year field trials on the influence of seed density and sowing date on the crop loss did not result in consistent effects on the different cropping practices. In 2002 the yield loss was lower in early sown plots than in plots sown at the normal sowing date. In the following year a similar effect could be observed for the cultivar Prince, but for the hybrid cultivar Talent the yield loss in early sown plots was higher than in normal sown plots. Due to an optimized inoculation method, disease incidences were higher in 2003 than in 2002. In 2002 the disease incidence was higher in plots of the normal sowing date whereas in 2003 early sown plots showed higher disease incidences (data not shown).

Sowing time can influence the disease incidence in natural infections with changes in coincidence of flowering/falling of petals and the availability of ascospores. The seed density affects the micro-environmental conditions in the plant cover and thus the conditions for *Sclerotinia* infection (TURKINGTON and MORALL, 1993).

As the *Sclerotinia* infections in our field trials were caused by artificial inoculations, the influence of sowing time and seed density on disease incidence can be disregarded. One-year field trials reported from China indicate higher disease incidence in early sown plots than in later sown plots, but plant density reportedly only had a slight influence on disease incidence (HU et al., 1999). The study of NORDIN et al. (1992) on the influence of three different seeding rates on the spread of *Sclerotinia* disease in spring-sown rapeseed shows contrasting trends in different field trials.

***Cost-effectiveness of fungicide applications against* S. sclerotiorum**

On the basis of a dataset from official field trials of the German state extension services, containing 855 individual fungicide treatments from 1991 to 2003, a validation of the cost-effectiveness of fungicide treatments against *Sclerotinia* stem rot was performed. For the preceding decade (1981 to 1990) similar investigations have been carried out by WAHMHOFF (2000).

A fungicide treatment during flowering (GS 61-67) achieved an average yield benefit of 2.41 dt/ha equivalent to 5.22 %. In the study by WAHMHOFF (2000), an average additional yield benefit of 2.96 dt/ha (= 8.3 %) was recorded for the years 1981 to 1990.

Fungicide applications in spring (GS 18-60) and combined applications (autumn/winter + spring and autumn/winter + spring + flowering) resulted in higher yield benefits than a single treatment during flowering. However, the yield benefits of these treatments can not only be attributed to *S. sclerotiorum* control as they also affect pathogens like *Phoma lingam*, *Botrytis cinerea* and *Alternaria brassicae* and also produce growth regulation effects.

The costs of a fungicide treatment and the additional yield benefit due to the treatment were considered to calculate the cost-effectiveness. The cost-effective yield gain from a fungicide treatment during flowering amounted to 3.32 dt/ha. According to Lor (1995) an additional yield of 6-7 dt/ha is necessary to cover the costs for a fungicide treatment in rapeseed. Analysis of the data set shows that only 33 % of the fungicide treatments were therefore economically efficient. The percentage of cost-effective fungicide treatments in 1981 to 1990 was 27 % (WAHMHOFF, 2000) and thus largely in accordance to the succeeding decade.

An average reduction of *S. sclerotiorum* incidence from 20.1 % to 7.7 % was achieved by a fungicide application during flowering. This corresponds to an average efficacy of control of 61.7 %. According to WAHMHOFF (2000) the average efficacy of fungicide applications against *S. sclerotiorum* in 1981-1990 was 70 % and thus slightly higher than in 1991-2003. Figure 2 shows the correlation between disease incidence and yield effect of a fungicide treatment against *Sclerotinia* stem rot. The yield effect increases with enhanced level of disease incidence. The studies of WAHMHOFF (2000) and KRÜGER and STOLTENBERG (1983) also show this relationship between yield gain and *Sclerotinia* incidence. LANDSCHREIBER (2004) reports on clearly higher yield effects of fungicide treatments at middle to high disease incidences as compared to low *Sclerotinia* incidence in Northern Germany.

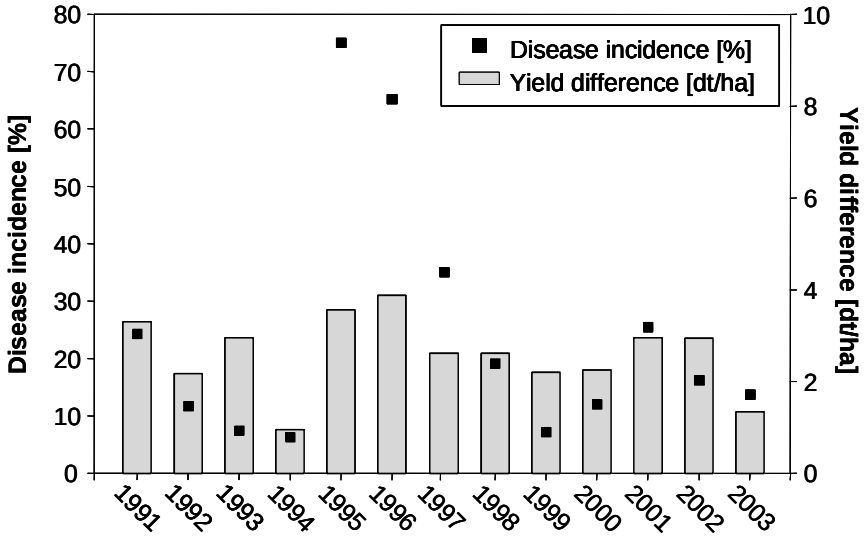

Fig. 2: Mean *S. sclerotiorum* disease incidence and yield effect of fungicide treatments during flowering, (data base: official field trials of the German state extension services, 1991-2003)

The analysis of cost-effectiveness of fungicide applications against *S. slcerotiorum* in relation to disease incidence by STEINBACH and BROSCHEWITZ (1997) in Northeastern Germany for the years 1981-1996 indicated that only in years with a disease incidence >4 % treatments were economically efficient. The study by KRÜGER and STOLTENBERG (1983) indicated that it was not until levels of >13 % *Sclerotinia* incidence that about 50 % of the fungicide treatments were economic and that only above 25 % disease incidence applications became cost-effective. The economic damage threshold for *Sclerotinia* stem rot proposed by these authors was at 15.5 % disease incidence.

This analysis shows the high percentage of ineffective fungicide treatments against *S. sclerotiorum.* Many growers conduct prophylactic fungicide treatments due to the lack of a reliable forecasting scheme for disease incidence and crop loss at the time of treatment. Due to this fact a damage threshold oriented forecasting system for *S. sclerotiorum* in winter oilseed rape is required to prevent inefficient fungicide applications.

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DNA polymorphism in *Sclerotinia sclerotiorum* isolates from oilseed rape in China

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Abstract: Sclerotinia stem rot casued by *Sclerotinia sclerotiorum* (Lib.) de Bary is a very important pathogen of oilseed rape in China and elsewhere. The aim of the study was to characterise individual isolates and fungal populations from Anhui and Qinghai Provinces in China and several isolates from Europe, using RAPD method. Additionally, the isolates were compared based on the sequences of the ITS1-5.8s-ITS2 region. The results demonstrated genetic variation of the isolates, both within China and between China and other countries. Most of the isolates from China formed one big cluster. The isolates from two distant provinces also differed from each other. However, a few isolates originating from China showed greater similarity to European isolates than to other isolates from China. The molecular methods used in this study showed genetic diversity in natural populations of *S. sclerotiorum* from China.

Key words: Internal Transcribed Spacer, ITS sequence, field population, sclerotinia stem rot, fungal isolate polymorphism;

Introduction

China is the first producer of oilseed rape in the world (7 million ha). The crop is grown mostly in provinces along the Yangtse river (mainly Hubei and Anhui). Rapeseed is cultivated in China for its high quality oil for human consumption. It also plays an important role in crop rotation and - due to its profitability - it serves as a good substitute of wheat. The perspectives of the non-agricultural use of this plant make it an even more attractive crop (Guan 1985). It is presumed that the acreage of rapeseed will expand in the near future. However, high intensity oilseed rape production is associated with greater pathogen inoculum density, with sclerotinia stem rot (white mould) as the most important problem for cultivation of rapeseed in China (Liu 2000, Li *et al.* 2001). The disease incidence on oilseed rape in Anhui Province differed from 10-30% in some years up to 80% of disease incidence. For example, the damage area in Anhui Province in 1998 was 70%, what means that at present sclerotinia stem rot is a prevailing disease (Zheng *et al*. 2000). The causal agent is a filamentous ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary. The fungus produces persistent soilborne sclerotia, which serve as the source of primary plant infection. The loss reported in China can reach 30% of seed yield, and the quality of the remaining harvested seeds is poor. As the pathogen can attack more than 400 agricultural and native plant species, the infested field may convey the disease to subsequent crops.

Although *S. sclerotiorum* is an important pathogen of rapeseed, the isolates originating from infected plants in China have not been closely studied and compared so far. The studies concentrated on the pathogenicity of the isolates (Liu *et al*. 2001) and the search for new sources of plant tolerance/resistance (Zhou 1994; Liu *et al*. 2003; Wang *et al*. 2004), with little interest in genetic characterisation of the individual isolates and their populations.

Random amplified polymorphic DNA (RAPD) markers have successfully been used in the analysis of genetic distances between populations (Hsiang and Mahuku 1999, Fulton *et al.* 1999, Tyson *et al*. 2002). In this study RAPDs were used to survey genetic variability among isolates from China and Europe. Polymorphic bands in isolates of *Sclerotinia sclerotiorum* from China were compared with several isolates collected from rapeseed fields in different locations in Europe. The isolates were further characterised by their sequence similarity of the ITS1-5.8s-ITS2 DNA fragment encoding the 5.8s ribosomal subunit and two segments of the Internal Transcribed Spacer. The sequence of the ITS region usually does not vary within one species (Barbee *et al*. 2003), but varies among different species and genera, and therefore allows differentiation of different taxons (Simmons and Freudenstein 2003). A recent study of ITS sequences of *Leptosphaeria maculans* and *L. biglobosa*, two other pathogens of oilseed rape worldwide (Morales *et al.* 1993 and 1995; Balesdent *et al.* 1998), showed variation of the ITS sequences and allowed differentiation of a multi-species complex as the cause of blackleg disease (Mendes-Pereira *et al.* 2003). The aim of this study was to investigate whether any intraspecific variation exists within the ITS regions of *Sclerotinia sclerotiorum* isolates.

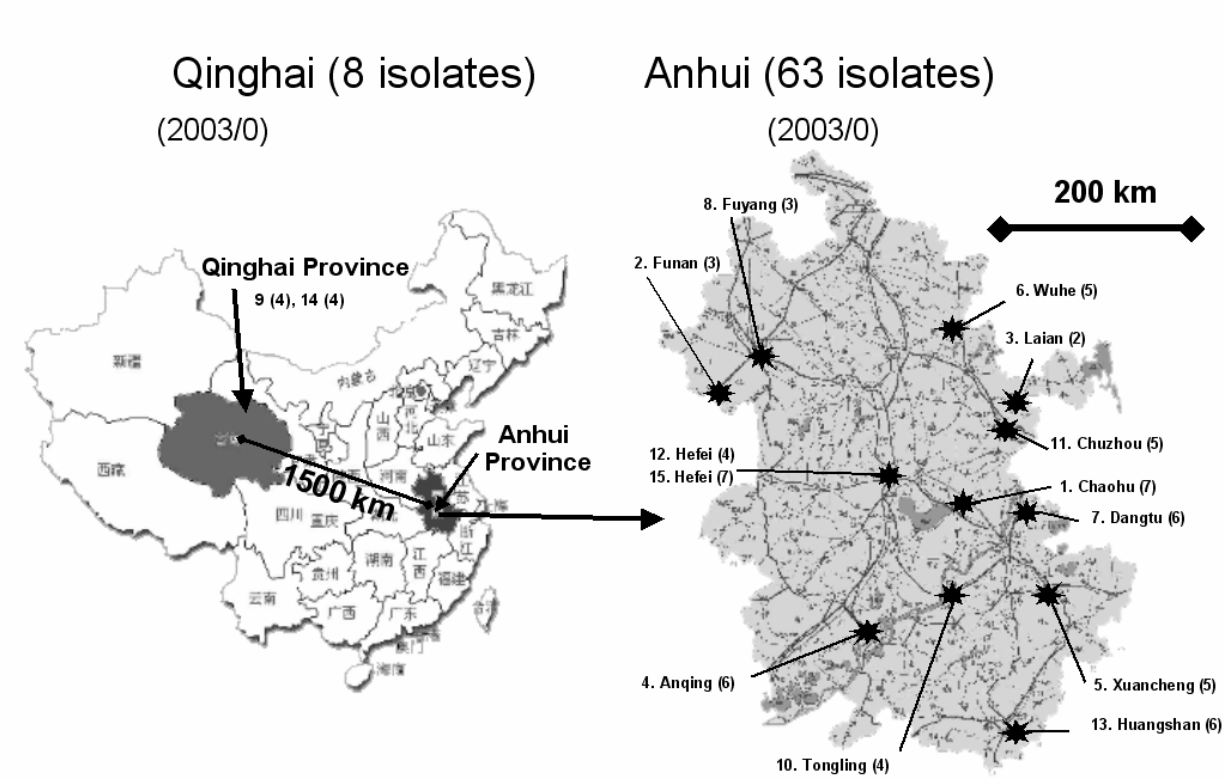
Materials and methods

The origin of Sclerotinia sclerotiorum isolates

The experiment used 87 isolates of *S. sclerotiorum* from oilseed rape, including 71 isolates from 15 localities in 2 provinces of China: Anhui and Qinghai (Figure 1). Most of the isolates (63 isolates, 88.7 % isolates from China, 72.4 % of the isolates studied) originated from the Anhui Province. The first collection took place in spring 2000 in one locality, a rapeseed field in Hefei (7 isolates). The other 56 isolates from the Anhui Province were collected in spring 2003 from the following locations: Chaohu (7 isolates), Funan (3), Laian (2), Anqing (6), Xuancheng (5), Wuhe (5), Dangtu (6), Fuyang (3), Tongling (4), Chuzhou (5), Hefei (4) and Huangshan (6) (Figure 2). The isolates collected in 2000 and 2003 in Hefei came from the fields located within the 0.5 km distance. The location of the sampling places in the Anhui Province and the rough distances between the fields are presented in Figure 2. The isolates from the Qinghai Province were also collected in spring of 2000 and 2003 in places within the 1 km distance. Altogether, there were 71 Chinese isolates studied, all of them were obtained from infected plants of spring oilseed rape. The remaining 16 isolates represent three countries in Europe: Russia (9 isolates), Poland (5), and Austria (2). The isolates from Russia represent two distant regions of rapeseed cultivation, Byelogorka near St. Petersburg in north-west Russia, where spring type is cultivated (4 isolates) and Krasnodar in south-west Russia, where winter oilseed rape is cultivated (5 isolates). The isolates from Poland were also collected in different regions and seasons. The isolates were obtained from single sclerotia collected from infected oilseed rape plants. The details on the origin of the isolates used in this paper are presented in Table 1.

Conditions of RAPD analyses

For molecular analysis, DNA was extracted with the DNeasy® Plant Mini Kit (Qiagen) from three day-old mycelium of *S. sclerotiorum* isolates, grown in shaking liquid Czapek-Dox medium supplemented with yeast extract (2 g/L).



Figures 1 and 2. The location of the sampling places in China and in Anhui Province

For RAPD analysis, we used three randomly chosen primer sets OPC, OPJ and OPL from QIAGEN Operon, each set containing 20 decamer primers. The sequences of these primers are available at http://oligos.qiagen.com/stock/rapd_10mer_price.php. The primers were initially screened using DNA from five randomly chosen isolates. Seven primers, showing the highest number of unambiguous polymorphic bands, were chosen for further analysis. The RAPD reactions were performed under mineral oil, in 4.5 µL final volumes containing 0.3 µL of template DNA, 200 µM of dNTP, 1 µM of a primer, 0.5 U of Taq polymerase (QIAGEN) in 1×PCR buffer (QIAGEN). Amplifications were performed in a MiniCycler™ (MJ Research) using the following programme: initial DNA denaturation step 2 min at 94 °C, followed by 45 cycles of 30s at 94 °C, 1 min at 36 °C, and 2 min at 72 °C, with a final extension at 72 °C for 5 min. The RAPD-PCR products were stored at 4 °C. Amplified fragments were separated by electrophoresis on 2% agarose gel (Life Technologies) in 1.0×TBE buffer, stained with ethidium bromide and visualized on the digital gel documentation system Scion Image release Beta 3b (Scion Corporation). The polymorphic bands were scored and analysed by TREECON for Windows version 1.3b software (Van de Peer and De Wachter 1994). RAPD analysis was performed for all isolates listed in Table 1, with the exception of one isolate from Austria (AUS 110).

Table 1. The origin of the isolates studied in this paper

Country	Locality	Province	No of isolates	Symbol of isolates	Form of OSR	Year
China	Hefei	Anhui	7	Ch_15	spring	2000
	Chaohu	Anhui	7	Ch_1	spring	2003
	Funan	Anhui	3	Ch_2	spring	2003
	Laian	Anhui	2	Ch_3	spring	2003
	Anqing	Anhui	6	Ch_4	spring	2003
	Xuancheng	Anhui	5	Ch_5	spring	2003
	Wuhe	Anhui	5	Ch_6	spring	2003
	Dangtu	Anhui	6	Ch_7	spring	2003
	Fuyang	Anhui	3	Ch_8	spring	2003
	Tongling	Anhui	4	Ch_10	spring	2003
	Chuzhou	Anhui	5	Ch_11	spring	2003
	Hefei	Anhui	4	Ch_12	spring	2003
	Huangshan	Anhui	6	Ch_13	spring	2003
	Xi'ning	Qinghai	4	Ch_14	spring	2000
	Xi'ning	Qinghai	4	Ch_9	spring	2003
Russia	Byelogorka	North-West	4	P	spring	2003
	Krasnodar	Kuban	5	K	winter	2003
Poland	Wierszczyca	Lubelskie	1	Sc-5	winter	1994
	Kalinowa	Łódzkie	1	Sc-6	winter	1994
	Bezek	Lubelskie	1	Sc-7	winter	1994
	Pępowo	Wielkopolskie	1	Sc-13	winter	1995
	Kąty Wrocławskie	Dolny Slask	1	Sc-18	winter	1995
Austria	Vitis	Niederösterreich	1	AUS-110	winter	1998
	Limpfings	Niederösterreich	1	AUS-115	winter	1998

Analysis of ITS fragment sequences

For analysis of polymorphism within the ITS region, we amplified a DNA fragment consisting of the 3' end of the 18s rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 5' end of 28S rRNA gene. Primer WIRZ G1 (5'-GTAACAAGGTTTCCGTAGGTG-3') was designed as a forward primer for PCR amplification and sequencing and was based on the *Leptosphaeria maculans* Leroy 18s rRNA gene (Morales *et al.* 1995). The reverse primer PN10 (5'-TCCGCTTATTGATATGCTTAAG-3') (Balesdent *et al*. 1998) was used both for PCR amplification and sequencing and both primers were purchased from QIAGEN Operon. Amplifications were performed under mineral oil, in 4.5 μL final volumes containing 0.3 μL of template DNA, 200 μM of dNTP, 2 μM of a primer, 0.5 U of Taq polymerase (QIAGEN) in 1×PCR buffer (QIAGEN). The reaction was performed in a MiniCycler™ (MJ Research), according to the following program: denaturation step at 94 ^{0}C for 2 min, followed by 45 cycles of 30 s at 94 ^{0}C, 30 s at 60 ^{0}C and 1 min at 72 ^{0}C, with a final extension at 72 ^{0}C for 5min. The amplified ITS fragments were stored at 4 ^{0}C. PCR products were cleaned from dNTPs and primers following a protocol available at http://www.genome.wustl.edu/tools/protocols/finishing/finVI.pdf. Sequencing of the ITS fragments was carried out using a Perkin Elmer ABI Prism 310 Genetic Analyzer. The sequencing reaction was done using an ABI PRISM BigDye V3.0 Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq DNA Polymerase following the manufacturer's instructions. DNA sequences were viewed with

Chromas 1.43 (Connor McCarthy) and compared to each using Clustal X (1.81). A BLAST search was done to compare the sequences with sequences available at GeneBank and the EMBL Nucleic Acid Database. The sequence of the ITS1-5.8s-ITS2 fragment was studied for all isolates.

Results

RAPD analysis

The number of unambiguously amplified polymorphic bands ranged from 1 (OPC-15) to 17 (OPC-02). The frequency of polymorphic bands differed from 1.2 % (a polymorphic band present in 1 isolate out of 86) to 98.8 % (the same polymorphic band amplified for 85 out of 86 isolates studied). Seven primers of the 60 primers initially screened were chosen for the analysis using all isolates, except AUS-110 from Austria. Primer OPC-02 generated the highest number of polymorphic bands (17), primer OPL-11 generated 11 polymorphic bands, and the primers OPL-12, OPJ-14, OPJ-13, OPC-04 and OPC-05 amplified 7, 6, 5, 3 and 1 polymorphic band, respectively. In total, 49 polymorphic bands were found. They allowed an investigation of the relationships between isolates using TREECON software. Some polymorphic bands (11, 22.4 %) were present only in isolates from China (OPC-02 bands 1, 4, 5, 11 and 20; OPJ-14 - bands 1, 2 and 4; OPC-04 - band 2; OPL-11 - band 6 and OPL-12 - band 4). Summarising, 11 out of 49 polymorphic bands (22.4 %) were found only in the isolates from China. Most of these bands (8 out of 11, 72.7%) were present only in the isolates from Anhui Province, 2 bands (18.2 %) were characteristic for the isolates from Qinghai Province and one band (9.1%) was present in some isolates from both provinces. Polymorphic bands amplified with the OPC-04 primer were found in numerous isolates (47 out of 63 isolates from Anhui Province, 74.6 %), whereas a polymorphic band obtained with the OPL-11 primer was found in one isolate only (Ch_1-2). There were no polymorphic bands common for the Chinese isolates only, but there was one polymorphic band (OPC-02 - band 6) observed in isolates from the Qinghai Province only. For European isolates, there was one polymorphic band found in 2 Polish isolates (OPC-02 - band 8) only, one band (OPC-02 - band 2) found in one Russian isolate (K11) only and two bands characteristic for European isolates (OPC-02 - band 3 for two Polish and one Russian isolate; OPJ-13 - band 2 found in one isolate from Russia and one from Austria). Primers OPC-15 and OPL-12 amplified one band each and these bands were found in all isolates from Europe, but also in numerous isolates from China (51 and 30 isolates out of 71 studied, respectively). A detailed list of the polymorphic bands amplified with the seven OP primers is presented in Table 2.

The RAPD method we used allowed differentiation of the 86 isolates studied into 66 groups, based on 49 polymorphic fragments amplified with the chosen primers. All but 10 isolates from China (85.9 %) formed one big cluster (Figure 3). The remaining 10 isolates from 5 localities in Anhui Province belonged to three clusters, together with one isolate from Poland and two from north Russia (Byelogorka). Most of the isolates (82.5 %) from Anhui Province clustered into one group. The isolates from Qinghai Province collected in 2000 and 2003, belonged to the cluster of isolates from China, but formed a distinct group. Based on RAPD analysis, the most distinct of the remaining isolates was isolate AUS115 from north-east Austria. The highest level of polymorphism was recorded for the isolates from two localities in China (Anqing and Wuhe), and the isolates from Poland and north-west Russia (St. Petersburg). Isolates from Qinghai Province (China) and Krasnodar (south-west Russia) formed distinct clusters. However, the isolates within these groups showed considerable polymorphism. The RAPD analysis showed no polymorphism among the isolates collected in

Hefei in 2000, they were all identical, with the exception of one isolate (Ch_Hf19), differing by one band (OPC-04 band 4). The isolates of *S. sclerotiorum* from Hefei, collected in 2000 differed morphologically from the remaining isolates studied (Sun 2003). They produced abundant light beige mycelium and big sclerotia formed in the PDA medium rather than on the medium, as was the case with the remaining, typical isolates. The RAPD analysis did not reflect the morphological difference of these isolates. The isolates were in one cluster, but highly related to the remaining isolates from China. The isolates from Hefei 2003 showed polymorphism and all isolates were different from those originating from Hefei 2000. The isolates from Hefei collected in 2003 were identical to other *S. sclerotiorum* regarding morphology.

Table 2. The frequency of polymorphic bands in *S. sclerotiorum* isolates from China and Europe

	17																	3			1	5				5				11											7						
Primer-Band/ Place	OPC-02-01	OPC-02-02	OPC-02-03	OPC-02-04	OPC-02-05	OPC-02-06	OPC-02-07	OPC-02-08	OPC-02-09	OPC-02-10	OPC-02-11	OPC-02-13	OPC-02-15	OPC-02-16	OPC-02-17	OPC-02-18	OPC-02-20	OPC-04-01	OPC-04-02	OPC-04-03	OPC-15-01	OPJ-13-01	OPJ-13-02	OPJ-13-03	OPJ-13-04	OPJ-13-05	OPJ-14-01	OPJ-14-02	OPJ-14-03	OPJ-14-04	OPJ-14-05	OPL-11-01	OPL-11-02	OPL-11-04	OPL-11-05	OPL-11-06	OPL-11-07	OPL-11-08	OPL-11-09	OPL-11-10	OPL-11-11	OPL-11-12	OPL-12-01	OPL-12-02	OPL-12-03	OPL-12-04	OPL-12-05	OPL-12-06	OPL-12-07
1. Chaohu				3						6		7				1	1	7	5	7	6	7		7	1	2	3	1	1		7			2	1	1				7		7	5	1	1		
2. Funan				3						2		3				1		3	3	3	3	3		3	2		1		1		3					2			1	3		3					
3. Laian							2					2		2	2	2				2	2	2		2	2	2			2		2					2			2	2		2	2		2		
4. Anqing							4			5		3	4	3	4	4		6	2	6	5	6		6	4	2			4		6			1					6		6	5		4	3	4
5. Xuancheng	2			2			1		1	2		4			1	1	1	4	4	5	4	5		5	4	1	2	1	1		5	2	1	4		1				5		5	5	3	1		1
6. Wuhe	1			2			2			4		3	4	2	2	2		5	3	5	4	5		5	3		1		2	1	5			2		1				5	1	4	3	2	3	2	2
7. Dangtu	1			4			1			5		5			1	1		5	5	6	6	6		6	5	1			1	1	6	1		2		3				6		6	3	1	1		1
8. Fuyang				2						1		3				1		3	3	3	2	3		3	2	2	1	1		1			1		2				3		3	1		1		
10. Tongling	1			3					1	2		4				1		4	4	4	2	4		4	3		1		1		4			1						4		4	2				
11. Chuzhou				2		1			1	3		4	3					5	3	5	2	5		5	3	1	1			1	5	1		3		4				5	1	4	3	1	1		
13. Huangshan				2					2	3		6	4					6	4	6	4	6		6	2	2		1			6	2		1		2		1		6		6	4	2			
12. Hefei				2								4	1					4	4	4	3	4		4		2			1	1	4			2		3		1		4		4	2	1	1		
15. Hefei				7						7		7						7	7	6		7		7					7		7					7				7		7			7		
9. Qinghai					4	2				4	3	1	4			2		4		4	4	4		4	4	4					3			4			2			4		4		1	4	1	
14. Qinghai					4					3	1	1	4			2		4		4	4	4		4	4	4					3			4			1			4		4	1		4	1	
China (71)	5			32	8	3	10		5	47	4	57	24	7	10	18	2	67	47	70	51	71		70	39	23	10	4	12	14	3	69	6	1	30	1	29	3	2	2	1	71	2	69	36	12	30	7	8
Russia (9)		1	1			1	4		5	3		6	3	3	3	3		7		6	9	8	1	9	5	6			3		1	5		3	2		5		3	5	8	4	5	4		9	2	1
Poland (5)			2			1	1	2	2	2		4	5	1	2	1		5		5	5	5		5	3	3			1			5	1		5		2		1		5	3	2	2		5		
Austria (1)						1				1		1	1	1	1	1					1		1	1	1	1			1		1	1		1		2			1	1			1	1		1		
Total (86)	5	1	3	32	8	6	15	2	12	53	4	68	33	12	16	23	2	79	47	81	66	84	2	85	48	33	10	4	17	14	5	80	7	5	37	1	31	8	3	6	6	85	9	77	43	12	45	9	9
Present ONLY in	China (A)	Russia	Europe	China (A)	China (Q)	Poland					China (Q)						China (A)		China (A)			Europe					China (A)	China (A)		China (A)						China (A)								China				

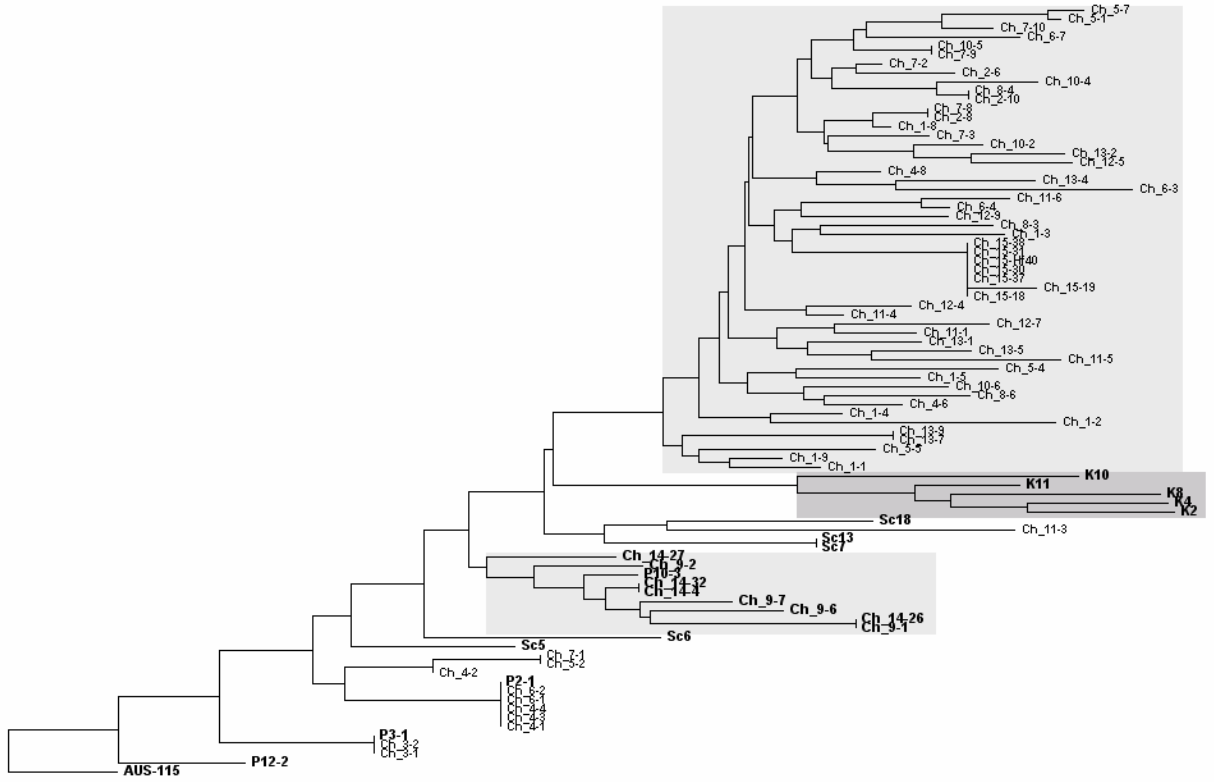


Figure 3. The similarity of *S. sclerotiorum* isolates based on DNA polymorphism studied with RAPD, dendrogram made by TREECON software (Van de Peer and De Wachter 1994)

Ch_4-8 (China)

ATTA|**CAGAGTTCATGCCCGAAAGGGTAGACCTCCCACCCTTGTGTATTATTAC⏩GTTTGTTG**
CTTTGGCGAGCTGCTCTTCGGGGCCTTGTATGCTCGCCAGAGAATATCAAAACTCTTTTTATT
AATGTCGTCTGAGTACTATATAATAGTTA|AAACTTTCAACAACGGATCTCTTGGTTCTGGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT**|T**
CAACCCTCAAGCTCAGCTTGGTATTGAGTCCATGTCAGTAATGGCAGGCTCTAAAATCAGTGG
CGGCGCCGCTGGGTCCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCGGTGTGCTTCTGCC
AAAA⏩GCCCAAATTTTCT|ATGGTTGACCTCGGATCAGGTAGGGATACCCGC

AUS-110 (Austria)

Figure 4. Sequence data of ITS1 and ITS2 (bolded letters) for *S. sclerotiorum* isolates studied

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Light leaf spot

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Field and controlled environment assessment of winter oilseed rape resistance to *Pyrenopeziza brassicae* (light leaf spot)

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Abstract: A field experiment at Rothamsted that assessed the development of light leaf spot on UK and Polish cultivars indicated that assessments of resistance done in the laboratory after incubation of plants were more precise than assessments done *in situ* in the field. Incubation of plants increased detection of symptomless infection. The cultivar Canberra, considered resistant (rating 8 of recommended list) also developed less light leaf spot in this experiment when assessment was done in the field using the HGCA RL protocol. However, incubation of plants for 5 days at 8^{o}C showed that it was heavily infected by *Pyrenopeziza brassicae*, similarly to the other cultivars used. For controlled environment test of resistance a conidiospore suspension concentration of at least 0.4 x 10^{5} ml^{-1} was necessary for the effective inoculation of 20-day-old oilseed rape seedlings.

Key words: *Brassica napus***,** resistance, assessment method, winter oilseed rape

Introduction

Pyrenopeziza brassicae is a pathogen that causes severe disease (light leaf spot) worldwide on many cruciferous plants, especially oilseed rape (*Brassica napus*). Light leaf spot on oilseed rape can cause yield losses of up to 1.5 t/ha, at an estimated annual cost of more than 30 million Euros in the UK. This loss occurs despite expenditure of more than 5 million Euros per annum on fungicide to control the disease.

Breeding for resistance to *P. brassicae* for is one of the possible strategies for the control of light leaf spot. There are differences in resistance to *P. brassicae* of oilseed rape cultivars (Walker *et al*., 1995) and between breeding lines of *B. napus* with resistance genes introgressed from *B. oleracea* and *B. rapa* (Bradburne *et al*., 1999). There is evidence from changes in UK recommended list resistance ratings for resistance to light leaf spot that sources of resistance to light leaf spot in new cultivars are being overwhelmed in a few seasons (Anonymous, 2004). The cultivar Apex changed from a resistance rating of 5 in 1994 to 7 in 1996 and to 5 in 2001; the rating for Bristol was 5 in 1994, 3 in 1995 and 2 in 1997; the rating for Synergy and Escort have both shown a decrease in resistance rating of one point since 1999. However, for some cultivars resistance rating increased: Cobra from 4 in 1993 to 6 in 1995; and Falcon from 6 in 1995 to 7 in 1996. There is a need to improve methods for measuring components of resistance of winter oilseed rape cultivars to *P. brassicae*.

Materials and methods

Field experiment

A field experiment was sown on the Rothamsted farm in August 2002 and consisted of three replications of six cultivars. Three cultivars were bred for Polish climatic conditions (Bosman, Kana, Marita) and three were from UK recommended lists (Apex, Canberra, Recital). Assessments of light leaf spot were done every 4 weeks, beginning on 23 January, 2003. Both laboratory and *in situ* assessments were done (Table 1). For the laboratory assessments, 10 plants were sampled from each of 18 plots, incubated for 5 days at 8^{o}C in polyethylene bags (Fitt *et al*., 1998). The level of infection was measured by:

- estimation of the percentage area of the whole plant affected by light leaf spot (with spots or sporulation);
- counting the number of leaves on each plant and noting how many were affected;
- estimation of the percentage area of each leaf affected;
- estimation of the percentage area of each stem affected;
- estimation of the percentage area of all pods affected.

Assessment of light leaf spot *in situ* was done using the HGCA/NIAB protocol (Anonymous, 2004):

- assessment done by eye as a percentage of the leaf area of the whole plot affected by light leaf spot (plants were not dug up, nor were leaves removed),
- between five and eight assessments on each plot are done over the season.

Table 1. Assessments done on winter oilseed rape samples from the field experiment to com pare six cultivars in Delharding field Rothamsted, between January and June 2003

	Assessment								
	In situ [b]	Before incubation	After incubation (5 days at 8^{o}C in polythene bags)						
Date	whole plant	number of leaves affected	whole plant	% leaf area affected	number of leaves affected	%plants with stems affected	% stem surface affected	% plants with pods affected	% pod surface affected
23.01.03	no	yes	no	no	yes	no[n]	no[n]	no[n]	no[n]
26.02.03	yes	no	yes	yes	yes	no[n]	no[n]	no[n]	no[n]
26.03.03	yes	no	yes	yes	yes	yes	yes	no[n]	no[n]
23.04.03	yes	no	yes	yes	yes	yes	yes	no[n]	no[n]
22.05.03	yes	no	yes	yes	yes	yes	yes	yes	yes
27.06.03	yes	no	yes	no[a]	no[a]	yes	yes	yes	yes

[n] organ not yet developed

[a] all leaves abscised

[b] HGCA/NIAB assessment

Controlled environment experiment

In a controlled environment experiment, resistance of 28 cultivars to *P. brassicae* was tested. Oilseed rape seeds from were sown in 2.5cm jiffy pots. One pot of each cultivar was randomly placed in each of four 35 cm x 21 cm plastic seed trays. Twenty days after germination, when they were at the four leaf stage, plants in each tray were sprayed with a

different concentration of *P. brassicae* conidia ($0.4x10^6$, $0.4x10^5$, $0.4x10^4$ or $0.4x10^3 mL^{-1}$) produced from UK mixed populations, using an aerosol sprayer (Gilles *et al*., 2000). The experiment was replicated three times, making a total of 12 trays. Because of the possibility of interplot interference (dispersal of inoculum from those plants inoculated at high concentration to those inoculated at low concentration), trays were arranged in treatment blocks and placed on separate benches. Jiffy pots were placed in seed trays and stood in gravel trays filled with tap water. They were placed in the CE cabinet at 16^o C. Artificial day-length was maintained at 12 h at a light intensity of 190 µE m^{-2} s^{-2}. After inoculation, trays were covered with propagator lids sealed with polyethylene tape. Propagator lids were removed after 48 hours. Assessment was done 21 days after inoculation by estimating the percentage of the leaf surface area affected, by sporulation or showing other light leaf spot symptoms such as green islands, yellowing or puckering (Karolewski *et al*., 2002).

Results

Field experiment

In the January assessment, between 64% and 81% of leaves of all cultivars were infected by light leaf spot when plants were assessed immediately after sampling. After incubation for 5 days, the number of leaves infected increased to between 79% and 85% (Table 2**)**. All assessment methods showed severe light leaf spot infection in February. Light leaf spot severity declined in the field in March and April, but symptoms were still found in incubated samples. Light leaf spot severity increased again in May, with very high scores on leaves after incubation. The percentage of the whole plant affected by the disease declined in June, after abscission of the remaining leaves, but the area affected increased on the developing pods. Analysis of variance showed differences in resistance to *P. brassicae* between cultivars were significant (at $P < 0.05$) before stem extension, when all but one (whole plant assessment in the laboratory) assessment method was used (Table 2). After stem extension, all assessment methods, with the exception of the March *in situ* ($P < 0.001$) assessment, gave no difference between cultivars.

Controlled environment experiment

No differences were observed between cultivars in severity of light leaf spot symptoms when all four treatments were included ($P = 0.102$, 27 df). However, only a few plants sprayed at the lowest concentration developed any symptoms at all, so this treatment was omitted from the analysis. When only three treatments were analysed, again no significant difference in infection was found between the cultivars ($P = 0.346$, 27 df). There were significant differences ($P < 0.001$) in the severity of symptoms caused by different concentrations of conidiospore inoculum (Table 3).

Discussion

Analysis of data from the first year of the field experiment which assessed the development of light leaf spot on UK and Polish cultivars indicated that assessments done in the laboratory after incubation of plants are more precise than assessments done *in situ* in the field. The *in situ* assessment can be done without removing part or all of the plant from the field and has the advantage of being fast and can be done at the same time as other disease or agronomic assessments.

Table 2. Assessment of field resistance to *Pyrenopeziza brassicae* for Polish and UK winter oilseed rape cultivars.

Date sampled	cultivar (resistance rating)*	% whole plant affected (*in situ* [a])	% leaves affected before incubation	% whole plant affected after incubation	% leaf surface area affected after incubation	% leaves affected after incubation	% stem area affected after incubation	% pod area affected after incubation
29.01.2003	Apex (5)	-	81,1	-	-	85,0	-	-
	Bosman	-	73,8	-	-	81,7	-	-
	Canberra (8)	-	64,3	-	-	79,0	-	-
	Kana	-	77,0	-	-	80,9	-	-
	Marita	-	74,8	-	-	82,4	-	-
	Recital (6)	-	77,6	-	-	84,2	-	-
	na	-	na	-	-	na	-	-
26.02.2003	Apex (5)	80,0	-	56,8	42,6	82,1	-	-
	Bosman	58,3	-	56,7	43,4	87,4		-
	Canberra (8)	40,0	-	58,3	44,3	87,2	-	-
	Kana	58,3	-	52,0	32,2	67,3	-	-
	Marita	63,3	-	59,7	41,1	80,4	-	-
	Recital (6)	61,7	-	55,5	46,2	87,1	-	-
SED (10 df)		5,46	-	3,29	2,62	0,06	-	-
26.03.2003	Apex (5)	61,7	-	27,8	29,6	63,5	12,7	-
	Bosman	50,0	-	36,3	29,8	69,6	2,6	-
	Canberra (8)	26,7	-	26,5	27,5	69,4	11,4	-
	Kana	36,7	-	31,8	27,6	66,5	5,0	-
	Marita	51,7	-	37,3	27,6	64,7	7,2	-
	Recital (6)	36,7	-	38,3	31,1	68,7	8,3	
SED (10 df)		5,81	-	6,03	5,60	2,38	5,37	-
23.04.2003	Apex (5)	3,7	-	20,3	29,9	74,8	5,8	-
	Bosman	2,3	-	18,0	30,4	79,2	6,3	-
	Canberra (8)	2,3	-	25,4	33,3	76,2	10,7	-
	Kana	4,0	-	23,0	39,3	82,3	10,5	-
	Marita	2,3	-	22,0	35,1	75,5	10,0	-
	Recital (6)	3,7	-	26,0	37,2	79,0	15,0	-
SED (10 df)		0,92	-	2,54	3,29	7,76	2,29	-
			-					
22.05.2003	Apex (5)	33,3	-	29,2	25,2	90,4	5,8	0,2
	Bosman	30,0	-	28,3	26,8	92,4	7,4	1,1
	Canberra (8)	30,0	-	22,7	17,9	91,3	6,8	1,7
	Kana	26,7	-	28,8	28,9	94,7	10,2	1,0
	Marita	28,3	-	22,3	18,8	89,7	4,0	1,3
	Recital (6)	40,0	-	27,7	27,9	93,8	8,4	0,4
SED (10 df)		16,49	-	3,50	4,81	5,30	3,45	1,37
27.06.2003	Apex (5)	11,7	-	18,3	-	-	9,4	26,3
	Bosman	18,3	-	17,2	-	-	12,8	21,5
	Canberra (8)	13,3	-	13,5	-	-	11,9	16,9
	Kana	13,3	-	18,3	-	-	13,7	27,7
	Marita	11,7	-	15,3	-	-	12,5	18,0
	Recital (6)	18,3	-	19,2	-	-	13,4	28,6
SED (10 df)		4,45	-	5,73	-	-	2,75	10,03

[a] = HGCA/NIAB assessment; no assessment of the 3 Polish cultivars na = not analysed

*resistance ratings defined in Table 3

Table 3. Resistance of winter oilseed rape cultivars to *Pyrenopeziza brassicae*, assessed in controlled environment experiment on 20-day-old seedlings

		concentration of conidiospore suspension (spores mL^{-1})			
		0.4×10^6	0.4×10^5	0.4×10^4	0.4×10^3
Cultivar	Resistance	% of leaf surface affected by *P.brassicae* (means over 3 replicates)			
Apex	-	13	25	7	7
Aviso	5^N	12	22	15	2
Bosman	5^p	10	17	12	0
Bristol	-	13	10	10	3
Canary	3^N	10	15	13	2
Canberra	7^p	15	32	20	2
Capitol	8^N	10	25	18	18
Cobra	7^N	15	17	5	0
Columbus	5^N	8	13	7	0
Escort	5^N	8	13	18	2
Eurol	7^N	22	18	5	0
Express	7^N	8	22	7	0
Falcon	8^N	23	32	22	10
Jet Neuf	7^N	13	22	10	0
Kana	3^N	12	7	12	0
Lipton	3^N	22	22	12	7
Madrigal	6^N	13	23	13	5
Marita	-	20	7	7	0
Mohican	-	23	23	22	0
Norin	8^p	30	17	0	0
Pollen	8^p	3	18	13	0
PR4SW05	6^N	10	28	5	2
Recital	6^N	17	22	3	3
Shannon	3^N	18	18	15	0
Talent	8^p	27	10	12	3
Twister	6^p	20	15	17	0
Vivol	6^p	22	18	10	0
Zenith	-	20	27	3	0

For 4 treatments S.E.D. = 0.39 (27 df): for the 3 highest concentration S.E.D. = 0.43 (27 df)

^{N} - HGCA/NIAB resistance rating

^{P} - figure provided by Procolza (a French consortium of oilseed rape breeders)

The cultivar Canberra, considered resistant (HGCA recommended list rating 8) developed less light leaf spot than the other cultivars when assessment was done *in situ* using the HGCA protocol. However, after incubation for 5 days, Canberra was heavily infected by the pathogen, as the other cultivars used. Laboratory methods gave consistent differences in resistance to light leaf spot between cultivars, when plants were assessed before stem elongation. Thus, it seems better to assess resistance on leaves than on stems or pods. The incidence and severity of light leaf spot epidemics was weather dependent. Dry weather in April resulted in a low severity of light leaf spot, when assessed *in situ.* After incubation however incidence and severity was again moderate to high (depending on which assessment method was used), indicating that the pathogen was present in sub-cuticular tissue in all cultivars and awaiting wet conditions to sporulate. The controlled environment experiment using 28 winter oilseed rape cultivars showed that there was no significant difference between them in their resistance to *P. brassicae* at the young plant stage. *P. brassicae* sporulation was seen on all cultivars at all concentrations of inoculum, with the exception of the lowest. For

resistance tests, conidial concentrations of 0.4×10^3 and 0.4×10^4 mL^{-1} were less effective for inoculation of oilseed rape leaves than 0.4×10^5 or 0.4×10^6 mL^{-1}. The cultivars used in this experiment were diverse in that they have been bred over a 20 year period from lines produced by several companies. It is, however, probable that they all originate from a fairly narrow genetic base.

Acknowledgements

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Preliminary results on the use of quantitative PCR for assessing resistance to light leaf spot (*Pyrenopeziza brassicae*) in oilseed rape cultivars

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Abstract Resistance to light leaf spot (*Pyrenopeziza brassicae*) on oilseed rape is currently assessed in inoculated field plots for UK National List purposes. The techniques used are time consuming and prone to inaccuracy due to the limitations of visual recording in oilseed rape plots, and the possible cryptic nature of infection in some material. Published primer sequences for *P. brassicae* have been used to quantify the disease in extracts of infected leaves using real-time PCR with a LightCycler™ system. Preliminary results with twelve cultivars indicate some agreement between leaf area infected scores in the winter and spring and amounts of fungal DNA recovered from leaves. However, in some cases the results suggest high levels of fungal development in the absence of visual symptoms. These results will be discussed in relation to the potential for molecular techniques to provide improved methods for resistance evaluation.

Key words: resistance, light leaf spot, evaluation, PCR

Introduction

Light leaf spot, caused by the fungus *Pyrenopeziza brassicae*, is a major disease of oilseed rape in the UK, causing losses estimated at £20 million per year. Cultivar resistance is a major breeding objective, and it is evaluated as a character for the UK National List and Recommended Lists. Sustainable oilseed rape production for the future is heavily dependent on reduction of inputs to control diseases such as light leaf spot, but this relies on an accurate determination of disease resistance. Currently, this character is assessed by visual estimation of the extent of symptoms. This provides some indication of relative resistance, but may be complicated by the existence of cryptic infections. Even when sporulation occurs, the symptoms of light leaf spot are difficult to see and quantify, requiring time consuming plot examination.

Quantitative PCR offers a rapid and potentially very accurate means of assessing the degree of fungal infection in a plant. Specific primer sequences for *P. brassicae* have been identified, and published (Foster *et al.*, 1999, Calderon *et al*, 2002), and systems for extracting fungal DNA from plant material are available (Foster *et al.*, 2002). Comparative variety resistance could thus be evaluated by PCR, and since this would take into account all infection, not just sporulating areas, a more accurate description of resistance could be achieved. Such a system is also likely to reduce the costs of testing, by avoiding lengthy plot examination, and replacing multiple field trials with a single inoculated experiment.

This paper presents initial results on the extraction and quantification of *P. brassicae* DNA from leaf material of a number of oilseed rape cultivars, and compares this to visual symptoms of the disease.

Materials and methods

Extraction and quantification of* P. brassicae *DNA

Collected plant material was cut into small pieces and placed in a 50ml centrifuge tube, and sealed with parafilm. Several holes were made in the parafilm and the samples were frozen for 30min in a -70ºC freezer. The samples were then freeze-dried over-night at -50ºC and then lightly crushed and mixed before being freeze-dried for a further 24 hours. Tubes were sealed and the samples stored at -20ºC until needed. The samples were ground again using a pestle and mortar to a fine powder and passed through a 500 micron mesh. DNA was extracted from a 20 mg sub-sample using the DNAeasy extraction kit (Qiagen) with no modifications to the standard protocol. Using primers originally published by Foster *et al*. (2002) a 753bp fragment of *P. brassicae* DNA was amplified and quantified using a LightCycler.

All experiments were carried out using the standard reaction mixture of 2µl LC FastStart SYBR Green 1 Master Mix (Roche Diagnostics), 0.5µl of each primer (5µM stock), 3.2µl 3mM $MgCl_2$, 2µl DNA template and water to a volume of 20µl. Reaction mixtures were loaded into chilled glass capillary tubes by centrifugation at 700 g for 5 s. PCR cycling conditions were as follows: initial denaturation at 95ºC for 10 min (to activate the 'hot start' *Taq*DNA polymerase) followed by 40 cycles of: 95ºC for 0 s, 68ºC for 5 s and 72ºC for 30 s. PCR product accumulation was measured once during each cycle following the end of the extension step. For melting curve analysis a melting programme was run at the end of the amplification steps: 95ºC for 0 s, 65ºC for 10 s and a ramp to 95ºC at a rate of 0.2ºC/s. Fluorescence was monitored continuously during the ramp step. Replicate *P. brassicae* DNA standards of 2, 20, 200 and 2000pg were included in all reactions.

Sampling of trials and visual assessment

A set of cultivars with known resistance ratings for light leaf spot was selected to derive the relationship between fungal DNA content and symptom expression. Leaves were sampled from inoculated trial plots at intervals by taking 30 leaves per plot, choosing the lowest fully green leaf on each occasion (ie with longest exposure to applied inoculum). Plots were inoculated by distributing infected stem pieces (5-7 per m^2) collected from infected plants the preceding harvest, and also by spraying spreader rows of a susceptible cultivar with a conidial spore suspension (10^6 spores ml^{-1}). Leaves were assessed individually for the extent of light leaf spot symptoms, and then enclosed in polythene bags for 72 h to encourage development of visible sporulation before being assessed again. Leaves were then bulked into 3 lots of 10, before extraction as described.

Results and discussion

Leaf area infected with visual symptoms and sporulation increased after incubation on all three sampling occasions, often by over 100% (Table 1). Scores did not always reflect resistance ratings (Table 2) which are based on larger data sets and published in the UK Recommended List (Anon, 2003). For instance, cv Shannon has a rating of 4.3 (1 to 9 scale, where 9 is most resistant), and had the same level of disease at the third sampling time as cv Recital which has a rating of 5.7.

DNA of *P. brassicae* was recovered from all cultivars at each sampling time. Mean amounts ranged from 5.4 to 211.3 pg DNA per g of host tissue at the first sample time, 25.6 to 153.9 pg g^{-1} at the second, and 32.6 to 197.8 pg g^{-1} at the third sample. There was very poor correlation between visual symptoms either before or after incubation and amount of fungal DNA at both the first and second sampling times ($r = 0.08$ and $r = 0.13$ for DNA content and

Table 1. Mean % light leaf spot cover on sampled leaves before and after incubation

Cultivar	22.01.04		12.02.04		04.03.04	
	Pre-incubation	Post-incubation	Pre-incubation	Post-incubation	Pre-incubation	Post-incubation
Shannon	1.50	2.75	1.28	4.68	8.73	22.58
Expert	0.20	0.05	1.50	5.23	8.30	11.40
Fortis	0.63	2.21	0.33	3.28	9.10	18.00
Recital	0.20	0.78	0.60	2.75	8.58	22.50
Royal	0.13	0.25	0.73	3.03	6.65	10.55
Tequila	0.00	0.00	0.30	1.57	4.33	6.50
Winner	0.50	1.29	0.80	5.05	7.35	16.13
Canberra	0.05	0.20	1.95	3.63	6.65	14.10
Elan	0.00	0.00	0.00	0.10	0.78	1.88
Mendel	0.05	0.26	0.60	3.13	7.08	12.48
Caracas	0.00	0.48	0.55	2.50	4.05	11.18
Gospel	0.00	0.18	0.15	0.98	6.35	11.45

Table 2. Cultivar resistance ratings (1-9, 9= most resistant)

Cultivar	Rating
Shannon	4.3
Expert	6.0
Fortis	5.9
Recital	5.7
Royal	5.6
Tequila	6.7
Winner	6.6
Canberra	7.6
Elan	8.3
Mendel	6.4
Caracas	5.8
Gospel	5.7

visual symptoms after incubation). At the first sampling time, the cultivar with the highest level of visible symptoms (cv Shannon) had one of the lowest DNA contents (29.7 pg g^{-1}) whereas cv Tequila, showing no visible symptoms, had one of the highest (202.9pg g^{-1}). Though the level of fungal DNA increased in cv Shannon at the second sampling time, it was still lower than that seen in other cultivars which had less visible symptom development. A significant correlation was seen at the third sampling time ($r = 0.67$, $p = 0.05$ for DNA content and visual symptoms after incubation). However, some cultivars with lower visual scores still had relatively high levels of fungal DNA, eg cv Winner (16 % leaf area cover) had almost the same level of DNA as cv Shannon. (122 vs 126 pg g^{-1}). Nevertheless, cv Elan, which had a low level of visible symptoms throughout, had a consistently low fungal DNA content (5.8, 25.6, and 32.6 pg g^{-1}) at each sampling time.

The results obtained so far suggest that quantitative PCR to determine fungal DNA content may be a useful tool for evaluating cultivar resistance. Further work is needed to optimise sampling times, and to investigate cultivars where high DNA content appears to be associated with comparatively low levels of disease expression. The possibility that there may be some degradation of fungal DNA in susceptible cultivars after sporulation has occurred should also be examined.

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Blackleg, stem canker

Large-scale survey of race structure of *Leptosphaeria maculans* in France

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Abstract: Nine avirulence genes were identified recently in *L. maculans*, whose combination theoretically generates up to 512 different races of the fungus. *L. maculans* is a pathogen with a high evolutionary potential to adapt to novel resistance genes as illustrated by the recent breakdown of the resistance gene *Rlm1* in France, where virulent populations of the fungus became strongly established within three growing seasons only. This information illustrated the need for a better knowledge of the fungal populations in terms of race, in order to better use available sources of major gene resistance. The objective of the present study was to get information on *L. maculans* population structure in France, based on a large-scale, appropriate sampling of populations and analysis of the frequency of each of the nine *AvrLm* alleles. Experimental fields, planted with a 'trap' cultivar known to harbour no major resistance gene, were set up in 17 locations. Single-pycnidium isolates were collected from leaf lesions developed in Autumn 2000 and 2001. Only 11 races were identified in the 1787 isolates analysed. One race, virulent on *Rlm1, Rlm2, Rlm3, Rlm4* and *Rlm9* was highly prevalent, representing more than 64% of the populations. Regional disparities were evident at all scale analysed. Some virulent races, such as those harbouring *avrLm5*, were present before the introduction of the corresponding R gene. Consequences for the durable management of resistance will be discussed.

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Frequency of avirulence genes in field populations of *Leptosphaeria maculans* in Germany, UK and Poland

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Abstract: In September 2002 seven research groups from four European countries (UK, France, Sweden and Poland) began a joint EU-funded project, SECURE (QLK5-CT-2002-01813). The project aims to develop molecular tools and elaborate a mathematical model to deploy durable resistance of oilseed rape to stem canker - one of the most damaging diseases of oilseed rape worldwide. The infection cycle of the disease, caused by the ascomycete *Leptosphaeria maculans* begins when ascospores land on cotyledons and/or leaves of oilseed rape in autumn. This primary inoculum is the product of genetic recombination which results in a high degree of recombination of pathogenicity traits, including the avirulence genes which correspond to resistance genes in the host plant. At the present time, nine resistance genes are known against *L. maculans* in oilseed rape (*Rlm1-Rlm9*). One of the aims of the SECURE project is to study the race structure (= frequency and combination of avirulence alleles) of field populations of *L. maculans* in Europe (600 isolates from six locations).

The isolates characterised in this study originated from field experiments established in the autumn of 2002 at four locations throughout Europe; two in the UK (Boxworth and Rothamsted), one in Poland (Poznań) and one in Germany (Teendorf). The field plots were sown with the spring cultivar Drakkar which was used as a "trapping cultivar" as this cultivar is currently known to lack any resistance genes. Thus, all isolates within the pathogen population would be able to infect plants and develop leaf lesions. Infected leaves with leaf lesions were collected randomly at each location and single-pycnidial isolations were made from the leaf lesions. In total, 274 isolates has been studied (176 from the UK, 77 from Germany and 21 from Poland).

The identification of *L. maculans* races was performed by inoculating each isolate on cotyledons of the extended set of nine differentials which incorporated eight known resistance genes. Assessments were done using the IMASCORE scale 0-6, where a 1-3 result (small to medium size necrotic spots) was interpreted as resistant reaction (i.e. the isolate was avirulent on the cultivar) and a 4-6 score (grey-green tissue collapse without or with the fruiting bodies of the fungus) was recorded as a susceptible plant reaction (i.e; the isolate was virulent). The results of this study showed a similar race structure to that recently described in France (see Balesdent *et al*. in this bulletin), with a lack of isolates possessing the avirulent alleles *AvrLm2, AvrLm3* or *AvrLm9,* and the lack of virulent isolates on *Rlm6* or *Rlm7 (avrLm6* or *avrLm7 isolates).* The *AvrLm1* allele ranged from 1.3 % (Germany) to 17.6 % (UK), *AvrLm4* ranged from 1.3 % (Germany) to 6.8 % (UK) and *AvrLm5* ranged from 2.6 % (Germany) to 14.2 % (UK). No *AvrLm1, AvrLm4 or AvrLm5* isolates have been found in the population from Poland so far. The UK population was more variable than the German population, but this may have been because the sample was represented by more isolates collected from two locations, whereas the German population sample consisted of fewer isolates collected at one site.

Key words: oilseed rape, *Phoma lingam*, resistance genes, stem canker, cotyledon test;

Introduction

In September 2002 seven research groups from four European countries (UK, France, Sweden and Poland) began a joint EU-funded project, SECURE (Evans *et al*. 2003). The project aims to develop molecular tools and elaborate a mathematical model to deploy durable resistance of oilseed rape to stem canker - one of the most damaging diseases of oilseed rape worldwide. The infection cycle of the disease, caused by the ascomycete *Leptosphaeria maculans* begins when ascospores land on cotyledons and/or leaves of oilseed rape in autumn. This primary inoculum is the product of genetic recombination which results in a high degree of recombination of pathogenicity traits, including the avirulence genes which correspond to resistance genes in the host plant (Ansan-Melayah *et al*. 1998). At the present time, nine resistance genes are known against *L. maculans* in oilseed rape (*Rlm1-Rlm9*). One of the aims of the SECURE project is to study the race structure (= frequency and combination of avirulence alleles) of field populations of *L. maculans* in Europe (600 isolates from six locations) (Figure 1).

Materials and methods

The isolates characterised in this study originated from field experiments established in the autumn of 2002 at four locations throughout Europe; two in the UK (Boxworth and Rothamsted), one in Poland (Poznań) and one in Germany (Teendorf). The field plots were sown with the spring cultivar Drakkar which was used as a "trapping cultivar" as this cultivar is currently known to lack any resistance genes. Thus, all isolates within the pathogen population would be able to infect plants and develop leaf lesions. Infected leaves with leaf lesions were collected randomly at each location and single-pycnidial isolations were made from the leaf lesions. In total, 274 isolates have been studied (176 from the UK, 77 from Germany and 21 from Poland).

The identification of *L. maculans* races was performed by inoculating each isolate on cotyledons of the extended set of nine differentials which incorporated eight known resistance genes. Assessments were done using the IMASCORE scale 0-6, where a 1-3 result (small to medium size necrotic spots) was interpreted as resistant reaction (i.e. the isolate was avirulent on the cultivar) and a 4-6 score (grey-green tissue collapse without or with the fruiting bodies of the fungus) was recorded as a susceptible plant reaction (i.e. the isolate was virulent).

Results

The results of this study showed a similar race structure to that recently described in France ((Balesdent *et al*. 2004), with a lack of isolates possessing the avirulent alleles *AvrLm2, AvrLm3* or *AvrLm9,* and the lack of virulent isolates on *Rlm6* or *Rlm7 (avrLm6* or *avrLm7 isolates).* The *AvrLm1* allele ranged from 1.3 % (Germany) to 17.6 % (UK), *AvrLm4* ranged from 1.3 % (Germany) to 6.8 % (UK) and *AvrLm5* ranged from 2.6 % (Germany) to 14.2 % (UK). No *AvrLm1, AvrLm4 or AvrLm5* isolates have been found in the population from Poland so far. The UK population was more variable than the German population, but this may have been because the sample was represented by more isolates collected from two locations, whereas the German population sample consisted of fewer isolates collected at one site.

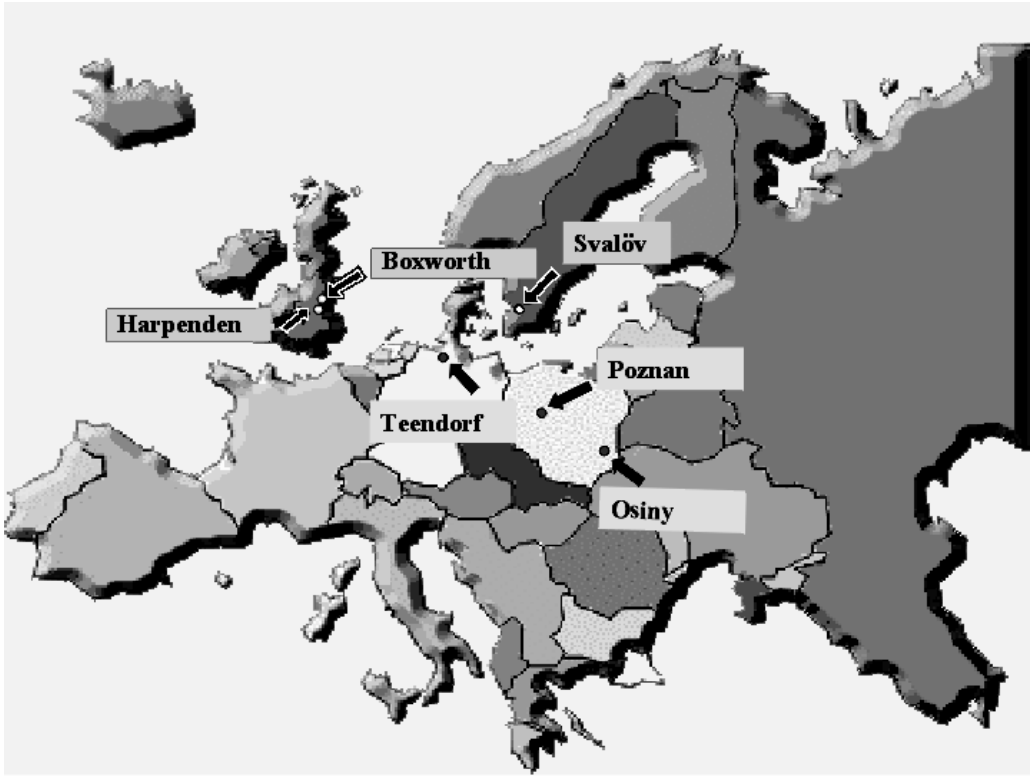

Figure 1. Location of experiment sites for studies of a race structure in field populations of *Leptosphaeria maculans* in Europe

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Field behaviour of oilseed rape genotypes carrying major resistance genes exposed to different *Leptosphaeria maculans* populations.

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Abstract: Six different major resistance genes (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm6, Rlm9*) were tested under field conditions using 7 oilseed rape lines to assess their level of field resistance against three different *Leptosphaeria maculans* populations in separate trials. Virulence allele frequencies in each of these populations were assessed under controlled conditions on a differential host set carrying the major genes leading to detect the presence of avirulence genes *AvrLm1, 2, 3, 4, 6* and *9* in the isolates. The level of disease under field conditions was assessed for each line by scoring leaf lesions in the autumn and stem base canker in June. From the data, no clear relationship was detected between the level of resistance observed in the field conferred by a specific *Rlm* gene and the relative frequency of the corresponding *AvrLm* gene within fungus populations. The result seems to vary depending on the *Rlm* gene considered.

Key words: Oilseed rape, *Leptosphaeria maculans*, major resistance genes, avirulence genes, fungus populations

Introduction

Leptosphaeria maculans (anamorph *Phoma lingam*), the causal agent of blackleg in crucifers, is one of the most damaging pathogens of oilseed rape in most regions of the world in which this crop is grown. The fungus survives on crop residues for at least 5 years. Airborne ascospores discharged from pseudothecia, mainly in autumn and winter in Europe, are the most important source of primary inoculum responsible for the occurrence of severe disease outbreaks (Gabrielson, 1983). Ascospores can be dispersed by wind over several kilometers, and cause field to field contamination (Gladders & Musa, 1980). Ascospores and pycnidiospores contaminate leaves, producing leaf lesions from which the fungus undertakes an internal systemic pathway to reach the base of the stem, where it initiates crown canker (Hammond & Lewis, 1987).

Two types of resistance to *L. maculans* exist in oilseed rape (*Brassica napus*) lines: a polygenic quantitative resistance (Pilet *et al*., 1998) and monogenic qualitative resistances conferred by several major resistance genes (Ansan-Melayah *et al*., 1998; Balesdent *et al*., 2001, 2002; Delourme *et al.*, 2004). The former resistance is considered durable (Rimmer & Van den Berg, 1992) whereas the latter resistance has been shown to be specific and non durable (Brun *et al*, 2000; Rouxel *et al*., 2003).

The level of field resistance of a line carrying a major resistance gene (*Rlm*) depends on the presence of the corresponding avirulence (*AvrLm*) or virulence (*avrLm*) gene in the fungus populations. The ratio of avirulence/virulence alleles needed within fungus populations to obtain a good level of resistance is unknown. The objective of the study is to assess whether

the level of field resistance conferred by different *Rlm* genes depends on the relative frequency of the corresponding *AvrLm* genes within the fungus populations tested.

Material and methods

Plant material

Under field conditions, six different major resistance genes were tested using the lines 'EurolMX' (*Rlm2, Rlm3, Rlm6*), 'Falcon' (*Rlm4*), 'Capitol' (*Rlm1, Rlm3*), 'SMX' (*Rlm1, Rlm3, Rlm6*), 'Eurol' (*Rlm2, Rlm3*), 'Samouraï' (*Rlm2*) and 'Darmor' (*Rlm9*). MXS is the original winter type oilseed rape line obtained by introgression of the resistance gene *Rlm6* from *Brassica juncea* into oilseed rape cv. 'Samouraï' (Chèvre *et al.*, 1997); it was backcrossed to cv 'Eurol' and 'S' line and advanced backcross generations were selfed to get the homozygous lines EurolMX and SMX. Eurol and EurolMX are near isogenic lines differing for *Rlm6* resistance gene. Darmor possesses a polygenic quantitative resistance expressed only at crown level.

The differential host set used under controlled conditions to assess virulence allele frequencies in each fungus populations allows to detect the presence of *AvrLm1, 2, 3, 4, 6* and *9* in the isolates: HD Maxol-SOO6 (*Rlm1*), Samouraï (*Rlm2*), 02-22-1-1 (*Rlm3*), Falcon (*Rlm4*), Darmor (*Rlm9*), Eurol (*Rlm2 Rlm3*), EurolMX (*Rlm2 Rlm3 Rlm6*).

Field experiment

Oilseed rape lines were grown in three field trials distant of at least 1 km, contaminated each by one fungus population previously obtained by field recurrent selection of *L. maculans* isolates on plant residue of either (i) Pop1, susceptible oilseed rape lines (without efficient *Rlm* genes), or (ii) Pop2, 'EurolMX' line, or (iii) Pop3, 'SMX' line. The infected rapeseed stem bases were scattered through the field at the three-leaf stage. Each field experiment was conducted in a randomised block design with four replicates. Each replicate was composed of four-row plots (6 m^2) of each line.

Leaf lesion assessment

Leaf lesions were scored on all leaves of every plant analyzed once in the autumn according to the development of the disease and 3 replicates were scored per line (*i.e*. 30 plants per plot, 90 plants analyzed in total per line).

Stem canker assessment

Stem canker severity was scored about two weeks before maturity (in June) on plants previously scored for leaf contamination. Sixty plants were scored per plot and per genotype. The plants were uprooted and stem canker scored based on observation of the internal and external extent of symptoms at the crown of each plant. A scale was used with class 1 corresponding to plants with no apparent symptoms and class 6 to those entirely broken down. A coefficient *i* (0, 1, 3, 5, 7, 9, respectively) was assigned to each class, and a disease index (DI) was then calculated for each plot as follows: $DI = (\Sigma_i\ i.ni)/N$ where *ni* is the number of plants in class *i*; and *N* is the total number of plant tested.

Fungal material

Single ascospore isolates were obtained from pseudothecia produced on stem base residue of Pop1, Pop2 and Pop3. Isolates were classified as *L. maculans* or *L. biglobosa* isolates according to their glucose phosphate isomerase patterns (Brun *et al.*, 1997).

Pathogenicity test

The cotyledons of 10 day old plants were slightly wounded in the center of each lobe with a needle and a 10-µl drop of a pycnidiospore suspension (10^7 pycnidiospores per ml) was deposited on each wound. There were therefore four inoculation sites per plant. Inoculum was produced as described by Brun (1994). A plastic cover was placed over inoculated plants to create an atmosphere with 100% relative humidity (RH). The plants were incubated in the dark at 20°C for 24h in a growth chamber and were then placed in an atmosphere with 80-90% RH (without plastic cover) with a 16 h photoperiod. The senescence of cotyledons was delayed by removing young leaves as soon as they appeared. Fourteen and 21 days after inoculation (dai), symptoms on cotyledons were scored using a 0 (no visible reaction) to 11 (total collapse of the tissue) rating scale. Each plant was considered to be a replicate and each isolate was used to inoculate 4 plants of each genotype sown in two replicates (giving 16 inoculation sites per genotype-isolate combination). A disease index of cotyledon (DIcot) was calculated according to the formula: DIcot = Σ (N_i x i)/N_t, where N_i is the number of inoculation sites with score i (0-11), and N_t the total number of inoculation sites (*i.e.* the number of well-developed cotyledon lobes). For each treatment, mean disease indices and standard deviations were calculated. Isolates were classified as (i) non aggressive when the DIcot $\leq$ 5 on all plant genotypes of the differential set at 21 dai (these isolates were not taken into account for calculating the frequency of virulence alleles) and (ii) for the aggressive ones, isolates were identified to possess an avirulent gene (*AvrLmi*) when DIcot + SD $\leq$ 5 at 14 dai and DIcot + SD $\leq$ 9 at 21 dai on the host possessing the corresponding resistance gene (*Rlmi*).

Statistical analysis of field data

For leaf lesion observations, average of the data obtained for each plot was calculated and then the standard error was calculated on mean of replicates. The stem canker DI data were surveyed by variance analysis in a randomized complete block design including the effect of the genotypes, the blocks and the genotype x block interactions per year using STAT-ITCF software (32) with $\alpha = 0.05$. The genotypes were classified using the Student-Newman-Keuls test (SNK test, $\alpha = 0.05$).

Results and discussion

Frequency of virulence alleles

The three fungus populations were very different for their genetic structure at the virulence level for *avrLm1*, *avrLm4* and *avrLm6* alleles (Table 1). Nevertheless, within the three fungus populations 100% a*vrLm3, 100% avrLm9* and close to 100% *avrLm2* isolates were detected.

Behaviour of oilseed rape lines

Exposed under field conditions to each of these fungus populations Eurol and Samouraï were always susceptible and Darmor resistant to stem base canker but susceptible to leaf lesions (Table 2). The differences of DI observed for each genotype between the three trials could reflect differences in the level of disease pressure. The good level of resistance of Darmor is confirmed at crown level in the three trials.

Table 1. Relative frequency (%) of isolates carrying the different virulence alleles (*avrLm*) within three *Leptosphaeria maculans* population.

L. maculans populations selected on	Virulence allele						*Number of isolates analysed*
	avrLm1	*avrLm2*	*avrLm3*	*avrLm4*	*avrLm6*	*avrLm9*	
Susceptible oilseed rape residue (**Pop 1**)	79.2	100	100	75	12.5	100	48
EurolMX residue (**Pop 2**)	16.3	97.7	100	88.4	100	nd	43
SMX residue (**Pop 3**)	100	100	100	36.9	100	100	19

Table 2. Frequency of plants with leaf lesions (%) in autumn and mean of disease index (DI) of stem base canker in June for several oilseed rape lines carrying different major resistance genes exposed to three different *Leptosphaeria maculans* populations under field conditions.

Oilseed rape lines		Eurol	EurolMX	Capitol	Darmor	Falcon	Samouraï	SMX	*P* value	CV %
Rlm:		*Rlm2 Rlm3*	*Rlm2 Rlm3 Rlm6*	*Rlm1 Rlm3*	*Rlm9*	*Rlm4*	*Rlm2*	*Rlm1 Rlm3 Rlm6*		
Pop 1	%	95.3 ±3.2	7.5 ±11.0	97.5 ±3.2	98.3 ±3.3	-	-	-		
	DI	7.8 ±0.2[1]	1.0 ±1.0	6.9 ±0.3	4.1 ±0.9	6.6 ±0.8	8.0 ±0.5	-		
	SNK groups[2]	AB	G	BCD	E	CD	A	-	0.0000	12.8
Pop 2	%	91.7 ±6.4	90.8 ±9.6	45.0 ±4.3	-	-	-	-		
	DI	7.1 ±0.9	8.3±0.2	4.9±0.4	2.1±0.1	4.7±0.6	6.4±0.6	-		
	SNK groups	B	A	C	D	C	B	-	0.0000	8.5
Pop 3	%				-	-	100 ±0.0	97.7 ±2.0		
	DI	-	-	-	2.9 ±0.4	2.4 ±0.7	6.2 ±1.3	5.4 ±0.3		
	SNK groups	-	-	-	D	D	BC	C	0.0000	13.6

[1] Standard deviation
[2] SNK test (α= 0.05)

Both at leaf and crown level, EurolMX was highly resistant (DI = 1.0) when it was exposed to a fungal population comprising 12.5% of *avrLm6* isolates (Pop1) but very susceptible (DI = 8.3) when there were only *avrLm6* isolates (Pop2).

The same level of disease was observed on Capitol (DI = 6.9) and Falcon (DI = 6.6) when the frequencies of *avrlm1* (79.2%) and *avrlm4* (75%) isolates were similar in (Pop1) trial with the highest disease pressure. Capitol exposed to a fungal population comprising 16.3% of *avrLm1* isolates (Pop2) displayed a DI = 4.9 whereas Falcon exposed to a fungus population comprising 36.9% of *avrLm4* (Pop3) displayed a DI = 2.4, even though in the

mean time the susceptible variety Samouraï had the same level of disease in the two trials (DI = 6.4 and 6.2, respectively). Moreover, EurolMX and Capitol exposed to two populations with similar frequencies of their corresponding virulence allele *i.e.* 12.5% of *avrLm6* (Pop1) and 16.3% of *avrLm1* (Pop2) displayed different level of disease *i.e*. DI = 1.0 and DI = 4.9, respectively.

From these results, no clear-cut connection can be established between the frequency of virulent isolates within fungus populations and an expected field level of blackleg disease or resistance for the oilseed rape lines carrying the corresponding resistance genes.

This first attempt will be completed by the results that will be obtained in the SECURE project where we follow the dynamics over the years of fungus populations (Pop1 and Pop2) and the level of disease of oilseed rape lines under different recurrent selective pressures.

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Identification of specific plant resistance factors to Phoma (*Leptosphaeria maculans*) among winter oilseed rape varieties: Variety testing and a framework towards durable management of resistance

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Abstract: Blackleg (*Leptosphaeria maculans*, phoma stem canker) is the main disease of oilseed rape. The growing of resistant cultivars is still the main control measure against the disease. The recent break down of a specific resistance gene widely used during the nineties, current knowledge, and genetic evidence from the fungus support a distinction between quantitative and specific resistances during genotype evaluations. This paper presents recent results of specific resistance identification in commercial varieties and suggests an integration with more classical tests to provide a framework to the durable management of plant resistances based on alternation and diversification of cultivars in time and in space.

Key-words: Oilseed rape, Blackleg, Disease resistance, durability, alternation.

Introduction

Phoma stem canker (*Leptosphaeria maculans*) is the most important disease of oilseed rape on a global scale. The epidemiology of the pathogen lends itself to control through agronomic practices. Stubble management after harvest is one of the most important aspects for the control of the disease and is a main target for soil tillage practices in oilseed rape (Gossende *et al.,* 2003). Fungicides are generally efficient, but often the length of the control period is not long enough to cover the whole period of spore dispersal so that control is not economically adequate. This is particularly the case during wet autumns when ascospore release can be longer than the two or three weeks control period afforded by a fungicide treatment. The main tool used to control blackleg is often the choice of the proper genotype. The first double low oilseed rape genotypes were mainly susceptible to the phoma stem canker. Plant breeders are looking for quantitative resistance as high as in simple zero cultivars like Jet Neuf or Darmor (Pilet *et al.,* 1998, 2001). Successful breeding was done during the 1990's, especially with respect to the use of the specific resistance gene *Rlm*1 for which the fungus population was mainly avirulent (Ansan-Melayah *et al.,* 1997). However, after several years of intensive use, this resistance appears to have broken down (Rouxel *et al.,* 2003). Important modifications of genotype behaviour were recorded as well as an increase of virulent isolates within the population. However, new specific resistances are being released in new cultivars and these are being released onto the market. Knowledge of the interactions between the pathogen and the plant induce a new approach in variety testing taking into account both specific and quantitative resistance factors in an attempt to promote the durable management of resistance. The aim of this paper is to present results from recent variety testing and to show how they can be integrated with a more classical approach to facilitate the durable management of resistance.

Materials and methods

Genotypes used are commercial varieties of winter oilseed rape registered in French or European catalogues. Certified seeds provided by breeders were used for greenhouse tests as well as for field experiments.

Specific resistance factors identification

Specific resistance factors were identified by doing cotyledon tests as described by Williams and Delwiche (1979). The use of eight reference isolates allowed us to check for the presence of nine specific resistance genes. Procedures for conservation, multiplication, fructification and preparation of the inoculum were previously describes by Ansan-Melayah *et al.* (1995). Young plants were first grown in the glasshouse and transferred into growth chamber before inoculation. For each strain/genotype association 2 x 10 plants were inoculated. Symptoms were assessed and recorded from 14 to 21 days after inoculation using a 6 level scale; a score of 1-3 = incompatible response, 4-6 = compatible response. A given genotype is considered as positive for a gene for gene resistance if at least 80% of the plants show an incompatible reaction for the virulence tested.

General behaviour against blackleg/phoma in the field

The varieties testing for general susceptibility against Phoma were done in four replicate randomized block designed experiments at several locations. These experiments were inoculated using local stubble. Leaf spots (incidence and sometimes severity) were assessed once or twice during autumn. Three weeks before harvest, stem cankers were assessed. For the final assessment, the G2 disease index was used. Here, 40 plants per plot were assessed and distributed among 6 severity groups affected by a severity coefficient to calculate the index (Pierre and Regnault, 1982). Several genotypes of different susceptibilities were used in the experiments as controls. Varieties qualifications (S-susceptible, IT-intermediate tolerance, VGT-very good tolerance) were done by combining data from experiments from different locations and often from different experimental years, taking into account, trial per trial, the relative disease index value compared to index values assessed for controls. Diseases index were also registered on agronomic variety testing trials for CETIOM national network when Phoma was present at a significant level through natural contamination. The number of plants collected to check the symptoms were often less than in the managed experiments, 10 or 20 plants per elementary plot. These last types of trials were added with the previous ones proportionally to the number of plants taken into account. For each variety the number of available results was rather high from 1 to more than sixty and also with 5 or 7 more trials coming from the registration process where specific trials for Phoma were also done.

Quantitative resistance identification

Currently, there is no test available to determine and evaluate quantitative resistance at the adult stage. Nevertheless, it is possible to produce an estimation indirectly if the field behaviour is not due to an efficient specific resistance which will make the quantitative resistance invisible. For such an approach, several pieces of information are needed: (i) leaf spot incidence (to be able to reject the hypothesis of an efficient specific resistance), (ii) the general field behaviour against the disease measured by the disease index G2 already described, (iii) the specific resistance genes already identified in the genotype for which the fungus populations are split among virulent and avirulent alleles. Much of this information was already available for French populations of *L. maculans* and these data suggest that virulent isolates for avrlm1 and avrlm4 are widespread (Rouxel *et al.*, 2003).

Results

Identification of known specific resistance genes

The following table presents the known identified specific resistance genes found in the commercial varieties tested. The majority of the tested varieties have at least one known specific resistance gene. Three varieties recently registered in the UK have the efficient *Rlm*7 specific resistance. This result is an important new data because the fungus populations are actually completely avirulent for this resistance. In contrast, we didn't find any variety with *Rlm*5, *Rlm*6 or *Rlm*8, which are the others specific resistances for which populations are still mainly avirulent.

Genotypes with the specific resistance *Rlm*1 were found in around 40% of the tested genotypes. Only 30% have *Rlm*4, and 10% of the total numbers have *Rm1* and *Rlm*4 together. Three specific resistances (*Rlm*2, *Rlm*3 and *Rlm*9) are not efficient in France because of dominant virulent populations. 40% of the tested genotypes have one or several of those specific resistance genes present. Their behaviour against the pathogen would be only determinate from a quantitative resistance or from an unknown specific resistance if no leaf spots were observed.

Field varietals evaluation

The classical procedure, during registration or for post registration, of *L. maculans* susceptibility tests is a field test in a phoma contaminated nursery. Two main variables are checked: the first one is the incidence of leaf spot during autumn, and the second the disease index G2 before harvest. Foliar spots are a useful epidemic indicator of the success of infection and of the annual epidemic pressure. In favourable epidemic conditions the leaf spots incidence is also dependant from the specific resistance genes present which act as a selective filter for the fungus population. Only virulent isolates are able to infect the plant. Then the absence of foliar spots on a genotype, in a favourable epidemic context, is an indicator of the presence of a specific resistance gene for which fungus populations are avirulent. We have varieties, all VGT, which are clearly in this category. Five varieties do not produce any leaf spots or only a very low number (Caiman, Olphi, Roxet, Hearthy and Es Astrid). When leaf spots were observed, they were often *L.* biglobosa or Alternaria spots. These varieties probably have efficient specific genes. For three of them Williams tests demonstrated that *Rlm*7 was present. For the two others the situation is less clear. Olphi has only *Rlm*1, and no known specific resistance genes were present in Es Astrid. For these varieties, we are trying to isolate virulent *L. maculans* isolates to check their virulence abilities. The hypothesis is that probably Olphi and Es Astrid have a new unknown specific resistance.

Collar observations three weeks before harvest provided a collar disease index G2 assessment for each trial and for each genotype. The general Phoma qualification determined from average index G2 values of the variety testing are given in Table 2.

The combined data from the previous results from each registration step allows an estimation of what could be roughly the level of quantitative resistance for each genotype. A large amount of the tested genotypes expressed a good level of quantitative resistance and then a good tolerance to Phoma.

Table 1: Results of specific resistance identifications in different genotypes, Results for older varieties were already published by Rouxel *et al.,* 2003

Genotypes				
with known efficient specific resistance *Rlm5, Rlm6, Rlm7*	with known partially efficient specific resistances *Rlm1, Rlm4*			without, or with one or several inefficient specific resistances among the known ones *Rlm2, Rlm3, Rlm9*
Rlm7	*Rlm1*	*Rlm4*	*Rlm1+Rlm4*	
Caiman, Hearthy, Roxet	Belcanto, California, Calypso, Canary, Cannelle, Capitol, Capvert, Cheyenne, Columbus, Constant, Expert, Geronimo, Licorne, Lutin, Makila, Maxol, Olphi, Parade, Salomont, Saturnin, Savannah, Tenor, Vivol	Adelie, Aligator, Bilbao, Colvert, Eleonore, Falcon, Lewis, Madrigal, Maestro, Milena, Nelson, Olifant, Pixel, Pollen, PR45W04, Synergy, Tosca,	Banjo, Cando Coronet Elite Explus, Extra, Liverpool Standing,	Aviso, Alesi, Akamar, Bristol, Campala, Caribou, Elan, Es Astrid, Eurol, Express, Frisbee, Goeland, Grizzly, Hecktor, Kintol, Kosto, Labrador, Mendel, Mohican, Navajo, Orlando, Pacific, Potomac, Récital, Sahara, Shannon, Smart, Symbol, Zenith

Discussion

With the determination of the presence of specific resistance genes it is now possible to have a more accurate varietal evaluation in addition to the information provided from observations of general behaviour under field conditions against Phoma. Recently, with clear break down of a specific resistance (*Rlm*1), has shown that such an approach is not enough (Rouxel *et al.,* 2003). Other break downs have been demonstrated experimentally (Brun *et al.,* 2002). Because of the instability of varieties, there is a risk for extension bodies that advice may not be appropriate thus causing a problem of “credibility” between the extension service and the

customer. However, the results and information produced during the current study allow us to distinguish specific and quantitative resistance. One is more exposed to the break down risk, the other much less. We were able to identify the specific resistances present in the different genotypes tested, and to roughly evaluate the level of quantitative resistance.

Table 2: Combined results of genotype evaluation against *L maculans* and indirect estimation of the level of quantitative resistance present in each cultivar. VGT : very good tolerance, IT : intermediate tolerance, S : susceptible.

General Behaviour (Index G2)	Specific resistances (*Rl*m)	Quantitative resistances	Number of Genotypes
VGT	No efficient *Rlm*	Good	13
VGT	*Rlm*1	Good	9
VGT	*Rlm*4	Good	5
VGT	*Rlm*1+4	Good	2
VGT	*Rlm*7	Unknown	3
VGT	Unknown *Rlm*	Unknown	2 ?
IT	No efficient *Rlm*	intermediate	9
IT	*Rlm*1	intermediate	11
IT	*Rlm*4	intermediate	6
IT	*Rlm*1+4	intermediate	3
S	No efficient *Rlm*	Low	8
S	*Rlm*1	Low	2
S	*Rlm*4	Low	5
S	*Rlm*1+4	Low	2

The first target of CETIOM is to promote genotypes with a good level of quantitative resistance. Our results show that a high number of varieties have already reached a high or intermediate level of resistance. This is an important result that illustrates the success of intensive breeding activities during the last fifteen years. Nevertheless new specific resistances could be very efficient and very attractive for economic reasons. Therefore, it is necessary to define specific means for the careful and durable management of available resistance genes to avoid break down. This is the second important objective. Diversification in space and time is the basic idea of our proposal to avoid strong selection pressures on the fungus population. On the other hand, at least two of the specific resistances (*Rlm*1 and *Rlm*4), are partially overcome. These could contribute partially to the general resistant behaviour of a genotype if the local fungus population has not become completely virulent. This could occur in some locations. Nevertheless today this type of contribution is probably low. Several cultivars having *Rlm*1 or *Rlm*4 are qualified as susceptible (see Table 1).

Results from Rouxel *et al.* (2003) suggest that in the absence of selection pressure, it would be possible for the population to revert back to a high proportion of avirulent isolates. Our third objective will be to try to stop population evolution towards virulence and then if possible to try to develop avirulence for *Rlm*1 and *Rlm*4. Around two thirds of the tested genotypes have one or both specific resistance *Rlm*1 and / or *Rlm*4. This approach allows us to develop groups of genotypes that produce the same kind of selection pressure and then distinguish groups on which the diversification of the selective pressure could be managed (Table 3). In group 1 we found the genotypes only with specific resistances that are already overcome and are therefore inefficient. Their control behaviour is only supported by quantitative resistances. Around 30% of the genotypes tested belong to this group where

diversity on others criteria could assume a wide choice for farmers with no constraints of management.

In group 2, genotypes with *Rlm*1 or *Rlm*4 are present. If this group and its subgroups are taken into account to avoid to maintain always the same selection pressure we may assume that this would avoid a complete break down of those two specific resistances and perhaps give them back a partial or complete efficiency.

The following table identifies groups with genotypes which have a new efficient specific resistance, already known (Group 3), or unknown and supposed through a lack of leaf spot (group 4). The careful use of genotypes belonging to these groups to avoid strong selection pressures, and alternation with genotypes from other groups may protect genotypes in this group from quick break downs. However, the low number of genotypes present in group 3 actually gives a limited choice with respect to encouraging diversification.

Table 3: Matrix which can be used as a tool to choose a cultivar and promote diversification of genotypes. The dark areas indicate that varieties belonging to both groups should not be grown in the following rotation or be side by side)

	Group 1	Group 2 *Rlm*1	Group 2 *Rlm*4	Group 2 *Rlm*1+4	Group 3 *Rlm7*	Group 4 *Rlm*X ?
Group 1						
Group 2 *Rlm*1						
Group 2 *Rlm*4						
Group 2 *Rlm*1+4						
Group 3 *Rlm*7						
Group 4 *Rlm*X ?						

Opportunities for diversification have to be connected with proper agronomic practices, especially with regard to stubble management in order to avoid increases of specific inoculums. Such practices are especially important for genotypes from groups 3 and 4.

Even in light of this scheme for diversification, there is still a huge amount of work to organise the application of this theory in the field. Specific discussions and collaborations with economic advisors are required to evaluate how such proposals could be use practically. Results from variety testing, general behaviour for Phoma resistance and diversification groups for each variety are available on the CETIOM web site which is regularly updated (www.cetiom.fr). In the near future (late 2004 or early 2005) a new tool to help farmers and economic advisors to choose an appropriate variety will be available on the web site, taking into account whether the interrogator is willing to entertain the present proposals of diversification to try to manage the choice in a durable way.

We have also to keep in mind that our proposals are only a preliminary step towards a durable management of resistance. More knowledge is required to be able to choose among more sophisticated and efficient strategies to decrease the break down risk.

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Feasibility of using quantitative PCR for assessing resistance to stem canker in oilseed rape cultivars

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Abstract: Stem canker (*Leptosphaeria maculans*) is the most damaging disease of oilseed rape in the major growing areas of the UK. Disease control through the exploitation of inherent resistance factors is now increasingly required in order to realise both the economic and environmental benefits desired by government, the industry and the public. Information on resistance is generated in "Value for Cultivation and Use" trials using an established technique in which stems are destructively sampled and the degree of symptom development assessed visually before harvest. The disease cycle of stem canker involves a long period of symptomless systemic growth in the autumn and winter, and the rate of systemic growth is likely to be highly correlated with the final expression of symptom severity. A molecular diagnostic method of measuring systemic growth would thus provide a highly specific and sensitive means of measuring cultivar resistance. This paper describes the development of a method for the detection of systemic growth of *L. maculans* in oilseed rape using quantitative real-time PCR, and its potential to replace the current field based assessments.

Key words: *Leptosphaeria maculans*, quantitative real-time PCR, resistance,

Introduction

Resistance to stem canker is an important characteristic of winter oilseed rape cultivars. The disease can cause yield losses of up to 40%, and recent average losses in the UK are estimated to be around £30 million each year (Fitt *et al.* 1997). The causal fungus, *L. maculans*, exists as two distinct pathotypes, virulent and avirulent. The former leads to the most damaging crown and stem cankers, while the latter induces less damaging pith necrosis. The disease cycle begins with the formation of leaf spots in the autumn. From these, the virulent pathotype grows systemically down leaf petioles to the stem base, where cankers are formed the following summer (Hammond *et al*. 1985). Manipulation of spray timings in trials with cultivars of differing canker resistances has indicated indirectly that in more resistant cultivars, rate of pathogen progress down leaf petioles is slower than in more susceptible cultivars (Thomas & Wedgwood 1998). Resistance is currently assessed in the UK in DEFRA funded trials by spreading infected oilseed rape stem debris in plots, and then assessing canker symptoms during June. Firstly, 30 stems are removed from each replicate plot, cleaned if necessary, and the degree of external symptom development assessed. The stems are then cut transversely in the leaf scar regions, and the degree of internal stem blackening recorded. The procedure is labour intensive, and thus costly, and though the technique is well established and used by plant breeders there are several technical disadvantages (Newman & Bailey 1987). Firstly, the symptoms of stem canker can be difficult to distinguish from those caused by stem infection of light leaf spot, and from insect damage caused by cabbage stem flea beetle. Secondly, the degree of internal blackening recorded often depends on the precise location of the transverse cut, and though longitudinal sectioning of the stem base area has been used experimentally, this would create severe practical constraints with the large sample numbers involved. Nevertheless, in recent years, visual assessments of canker development in

the UK cultivars have indicated that progress in breeding for resistance has been made (Thomas *et al*. 1999).

Molecular diagnostic techniques in plant pathology are being increasingly used in a wide range of applications including the evaluation of disease resistance. The technique offers the advantages of specificity, sensitivity and speed particularly where symptom development is ambiguous or difficult to judge visually. The development of real-time PCR has also made the use of quantitative PCR more accessible and reliable.

DNA primer sequences specific for the virulent and avirulent pathotypes have been published (Mahuku *et al*. 1995 & 1996), and have been used to quantify resistance to canker in seedlings of spring oilseed rape in Canada (Mahuku *et al*. 1995). However only two cultivars were assessed, and the technique used leaf tissue and not petiole tissue. The use of molecular diagnostic techniques such as these offers the possibility of evaluating resistance without the confounding effects of the factors described above. In addition, such techniques are likely to be high throughput and have a low unit cost.

This paper describes the potential for a molecular diagnostic approach to quantitatively evaluate cultivar resistance to stem canker by assessing the growth of the virulent pathotype in the leaf petioles.

Materials and methods

Collection of fungal isolates

The *Leptosphaeria maculans* isolates used in this study were obtained from throughout the UK from both leaf lesions and stem base cankers over a number of years (Table 2). Fungal isolates derived from single conidia were cultivated and maintained on V8 agar.

Collection of infected oilseed rape leaf samples

Ten oilseed cultivars on the UK Recommended List (2001) with a range of resistance ratings for stem canker were selected. 30 leaves were collected from each of three replicate plots in trials established at Boxworth, Cambridgeshire with the youngest fully expanded leaf selected regardless of whether there was a visible *L. maculans* lesions on 1st November 2001. Each leaf was frozen individually at –20ºC until later analysis. At the same time a further 30 cultivars with a range of resistance ratings for stem canker were selected. The cultivars were divided into five infection categories based on their performance in inoculated trials in the previous year with a number of cultivars selected from each group.

The 30 leaves collected from each plot were sub-divided into three batches of ten leaves for each of the cultivars. For each leaf the distance from the edge of the lesion to the base of the petiole was measured and divided into four, giving sections A – D with A being adjacent to the lesion. In this experiment only sections C and D were analysed. After the petiole tissue had been freeze dried and ground to a fine powder 10 mg of tissue was removed from the three replicates from each plot (labelled C or D 1-3) and combined to give a further sample (C or D 4). This gave nine primary samples and three combined samples for each cultivar.

DNA extraction from infected petiole tissue

The petiole tissue was cut into short lengths and frozen for 30 minutes in a -70ºC freezer. The samples were then freeze-dried over-night after which they were stored at -20ºC until needed. The samples were ground to a fine powder using a pestle and mortar, before the DNA was extracted from a 20 mg sub-sample using the DNeasy kit (Qiagen, Germany).

Preparation of standard mixtures of* Leptosphaeria maculans *DNA
The DNA isolated from an highly virulent (HV) and weakly virulent (WV) isolate was quantified using a fluorimeter and a series of samples were artificially created with varying proportions of each isolate in the following proportions: 100% HV, 80% HV and 20% WV, 60% HV and 40% WV, 40% HV and 60% WV, 20% HV and 80% WV, 100% WV.

***Detection and quantification of highly and weakly virulent* L. maculans in planta.**
Comparison of the primers originally published by Mahuku *et al*. (1996) and Xue *et al*. (1992) with recently available sequence data for *L. maculans* showed them to be potentially flawed. Use of this updated sequence information allowed the development of a new set of primers specific for the highly (HV) and weakly (WV) virulent types (Table 1). Primers HVF1 & HV26c are specific for the highly virulent type whilst WVF1 and WVR1 detect the weakly virulent type producing a 377 or 237 base pair product respectively.

Table 1. Sequences for primers developed for the detection of highly and weakly virulent *L. maculans* isolates.

Primer	Sequence
HVF1	5′-GTGGCGGCAGTCTACTTTGA-3′
HV26c	5′-GAGTCCCAAGTGGAACAAACA-3′
WVF1	5′-CCTTCTATCAGAGGATTGGT-3′
WVR1	5′-CGTTCTTCATCGATGCCAGA-3′

After optimisation of conditions for use of the primers in a Lightcycler all experiments were carried out using the standard reaction mixture of 2µl LC FastStart SYBR Green 1 Master Mix (Roche Diagnostics), 0.5µl of each primer (5µM stock), 1.6µl 3mM $MgCl_2$, 2µl DNA template and water to a volume of 20µl. Reaction mixtures were loaded into chilled glass capillary tubes by centrifugation at 700g for 5 s. PCR cycling conditions were as follows: initial denaturation at 95ºC for 10 min (to activate the 'hot start' *Taq*DNA polymerase) followed by 30 cycles of: 95ºC for 0 s, 62ºC for 5 s and 72ºC for 15 s. PCR product accumulation was measured once during each cycle following the end of the extension step. For melting curve analysis a melting programme was run at the end of the amplification steps: 95ºC for 10 s, 60ºC for 10 s and a ramp to 95ºC at a rate of 0.1ºC/s. Fluorescence was monitored continuously during the ramp step. As both primer pairs required similar conditions the possibility of multiplexing the reactions allowing the detection of both virulence types in the same reaction was investigated. The specificity of the primers was tested using two isolates whose virulence had been previously established using methods described by McGee & Petrie (1978). Isolate 1 (weakly virulent) and isolate 5 (highly virulent) were isolated from DEFRA trials.

Results and discussion

***Melting peaks of PCR products from highly and weakly virulent isolates of* L. maculans**
The expected products were obtained from the highly virulent and weakly virulent isolates (377bp and 237bp respectively) which gave melting peaks with T_m values of 85.61ºC and 82.74ºC (Figure 1). The products from these reactions were analysed on an agarose gel confirming that only a single product was produced with each of the primer sets. (Figure 2).

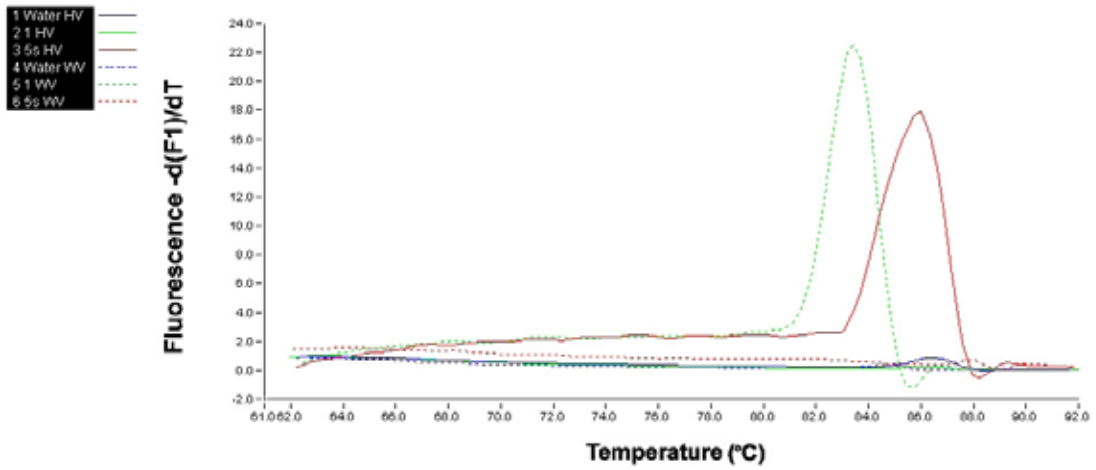

Figure 1. Melting peaks of PCR products from *L. maculans* isolates that are weakly virulent (green line) and highly virulent (red line).

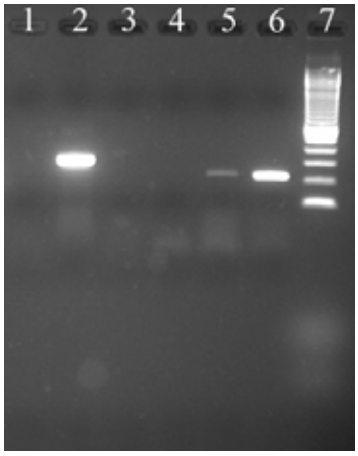

Figure 2. Agarose gel showing PCR product bands from different virulence primers. Lane1 water + HV, lane 2 isolate 5 + HV, lane 3 isolate 1 +HV, lane 4 water + WV, lane 5 isolate 5 +WV, lane 6 isolate 1 + WV, lane 7 DNA ladder.

Melting peak analysis of mixed* L. maculans *virulence populations

The DNA was amplified in a multiplex reaction in the presence of both primer sets and the resulting melting curve analysed. As the multiplex PCR is a competitive reaction, changing the relative amounts of each isolates DNA in the reaction mixture gave a pattern of melting peaks that reflected the proportions of the two virulences in the starting sample (Figures 3 and 4). The height of a peak was measured from the water control baseline to the point at which it crosses the T_m line of the 100% HV control sample (red vertical line in Figure 3), as fitted by the lightcycler software. The height of a peak was then calculated as a percentage of the 100% control sample. This was found to have a linear relationship with the original ratio of the mixture with a high degree of correlation ($r^2 = 0.9569$) (Figure 5).

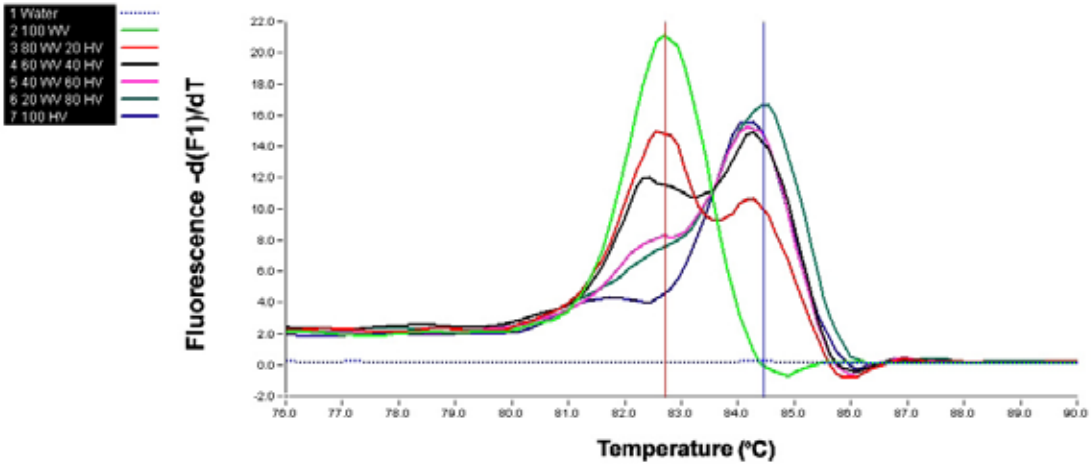

Figure 3. Melting peaks of PCR products from *L. maculans* isolates in various mixtures.

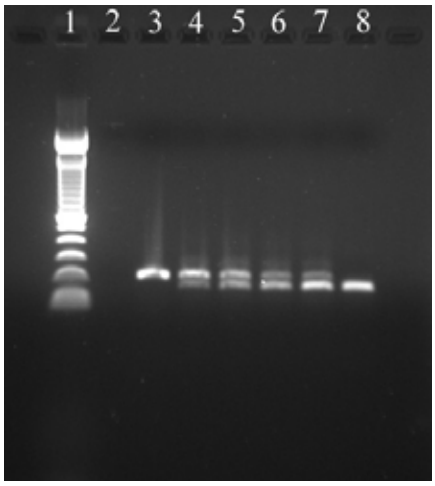

Figure 4. Agarose gel showing the relative intensity of PCR product bands from different virulence mixtures. Lane 1 DNA ladder, lane 2 water, lane 3 100% HV, lane 4 80% HV and 20% WV, lane 5 60% HV and 40% WV, lane 6 40% HV and 60% WV, lane 7 20% HV and 80% WV, lane 8 100% WV.

Analysis of* L. maculans *isolates of unknown virulence.

All isolates were tested with both primer sets and assigned a virulence category based on the fragment produced (Table 2). None of the isolates tested gave amplification with both primer sets confirming the specificity of the newly designed probes. The majority of isolates were found to be highly virulent.

Correlation between* L. maculans *DNA and field scores for ten oilseed rape varieties included in the UK recommended list.

Comparison of pathogen DNA concentration from the petiole sections C 1-3 with the five-year mean canker scores showed a non-linear relationship (Figure 6) with a correlation coefficient of 0.75. The C4 samples showed a similar relationship but with significantly lower correlation (0.59). This suggests that whilst the C4 sample for a given plot is representative of the C 1-3 replicates the analysis of the individual samples rather than a constructed mixture improves the reliability of the data produced. Petiole section D also showed a non-linear

relationship with the five-year mean, with a correlation coefficient of 0.72. Combining of the results for sections C and D did not significantly improve the overall correlation (0.77).

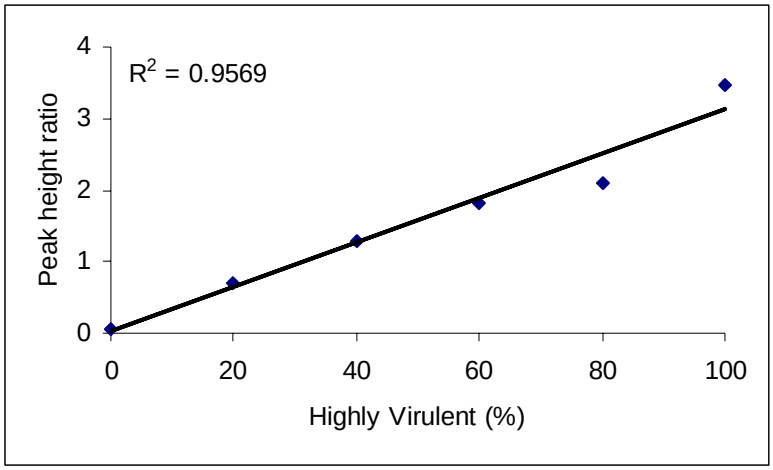

Figure 5. Relationship between original ratio of virulences in samples and resulting melting peaks of PCR products.

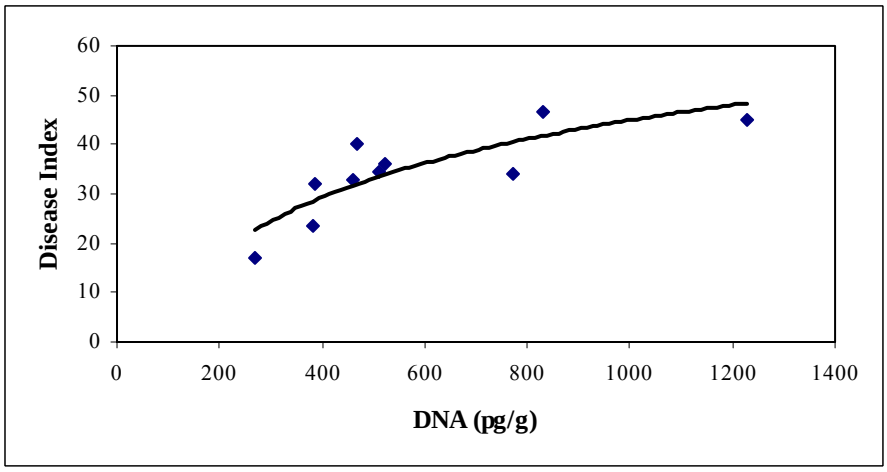

Figure 6. The level of *L. maculans* DNA in the petiole sections C 1-3 from ten oilseed rape cultivars compared to five-year field score mean.

Correlation between* L. maculans *DNA and field scores for 30 oilseed rape varieties

The use of the constituted C4 sample only, resulted in a poor non-linear relationship with a resistance data from the three previous trials to have contained these cultivars with a correlation coefficient of 0.48. This compares to the field scores from this trial which showed good correlation with the three trial mean with a coefficient of 0.88. As we had previously found that the use of the C4 sample gave a poorer correlation, the C 1-3 samples for nine cultivars were re-analysed. This significantly improved the relationship with the three trial average with a coefficient of 0.73.

Table 2. List of *L. maculans* isolates used in study and their virulence type.

Isolate Number	Collection Details Year	County	Cultivar	Virulence (HV / WV)
1	1996	Cambridgeshire	Express	WV
2	1997	Cambridgeshire	Synergy	HV
4	1996	Cambridgeshire	Gazelle	HV
5	1999	Cambridgeshire	Unknown	HV
6	1996	Hampshire	Unknown	HV
7	1999	Cambridgeshire	Unknown	HV
11	2000	Cambridgeshire	Unknown	HV
12	2000	Kent	Unknown	HV
13	2000	Cambridgeshire	Unknown	WV
14	2000	Cambridgeshire	Unknown	HV
15	1995	Cambridgeshire	Gazelle	HV
20	2002	Staffordshire	Escort	HV
21	2002	Gwent	Unknown	WV
22	2002	Yorkshire	Disco	HV
23	2002	Wiltshire	Shannon	HV
24	2002	Somerset	Escort	HV
25	2002	Suffolk	Courage	HV
26	2002	Cambridgeshire	Unknown	HV
27	2002	Warwickshire	Escort	HV
28	2002	Suffolk	Tequila	HV
31	2002	Lincolnshire	Winner	HV
32	2002	Staffordshire	Shannon	HV
33	2002	Cambridgeshire	Winner	HV
34	2002	Shropshire	Canberra	HV
35	2002	Rutland	Winner	HV
36	2002	Staffordshire	Escort	HV

Analysis of section D from all 30 varieties, whilst resulting in a correlation of 0.61 with the field scores from this trial had a poor non-linear relationship with the three trial mean (0.38). In previous studies (data not included) combining results for sections C and D resulted in an improvement in the relationship between pathogen DNA and field scores. This was not the case in this trial where the correlation coefficient was 0.48. Here however the C section data was limited to the C4 combined sample. Use of the nine varieties were all nine primary C samples had been analysed resulted in a significantly better correlation of 0.73.

Conversion of* L. maculans *DNA to a disease index using a standard curve

Using results from the 10 varieties in the recommended list trial the level of pathogen DNA detected in petiole sections C and D was converted to a disease index score. This resulted in a linear relationship with a correlation of 0.61 when compared to the three trial mean (Figure 7). When the conversion was limited to the nine NL2 varieties were the C section is represented by sections C1-3 and not the C4 sample only then the correlation improves to 0.73.

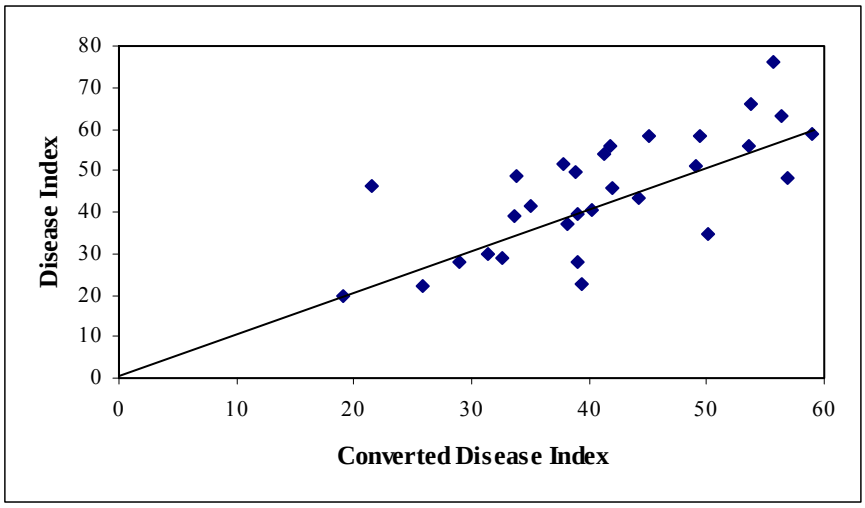

Figure 7. The level of *L. maculans* DNA in the petiole sections C and D from 30 NL2 oilseed rape cultivars converted to disease index compared to three trial field score mean.

Use of molecular probes to quantify *L. maculans* DNA in host tissue has the potential to provide an alternative method for the assessment of a varieties innate resistance to the development of stem canker symptoms. Use of multiplex PCR allows both *L. maculans* types to be distinguished and their relative proportions to be established. In the experiments detailed above use of real-time PCR detection of *L. maculans* DNA resulted in levels of correlation with bulked multi-year field scores that were of a similar order to that achieved using standard field assessments. However multiple DNA analyses are required for each plot to achieve the greatest level of correlation with field results.

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Durability of resistance, a modelling approach

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Abstract: Using ordinary differential equations, a model of durability of resistance was developed. The current model includes two strains of the pathogen (one virulent and one avirulent) and two crops (one resistant and one susceptible). This study focuses on the analysis of the model complexity and its effects on the simulation of the temporal evolution of both strains of the pathogen and hence on the durability of resistance. Successive levels of complexity were (1) the introduction of seasonality instead of continuity, (2) the introduction of two ways to define partial resistance and (3) the change from a polycyclic to a monocyclic disease with a single ascospore arrival at the start of the season. Early results suggest that no major differences in the temporal behaviour of the model simulations can be observed with increased complexity.

Key words: Ordinary differential equations, durability of resistance, model complexity, *Leptosphaeria maculans*

Introduction

Durability of resistance is a major problem in modern agriculture. Indeed, the introduction of resistant cultivars is a strategy widely used in the field to reduce the effects of diseases. Unfortunately, when a resistant cultivar is introduced into the field, it is only a matter of time before this resistance is broken down. However, the time this takes can be very variable. For instance, resistance of oilseed rape to *Leptosphaeria maculans* induced by the major resistance gene *Rlm*1 was broken down within three years after its introduction in the field in France (Rouxel et al., 2003).

In order to avoid such rapid break-down of resistance, modelling can play an important role. Indeed, as we explained in an earlier part of this work, we can first use models to discuss and compare different definitions of durability of resistance according to the objective one may try to achieve. Hence, we have already shown – and this is a result which would neither be simple nor quick to show from field experiments, at least because of the large number of values and parameters tested – that the optimum strategy to follow will be very dependant on the criterion used to define "durability of resistance". However, in this work, we concentrate on testing the effects of the model complexity on the simulations that it produces and the results obtained.

Material and methods

In this work, we use an ordinary differential equation (ODE) model based on the continuous model developed by van den Bosch & Gilligan (2003). We assume a logistic growth of the crop and a constant sowing density across seasons. At the moment, the model has two cultivars and two strains of the pathogen, hence leading to four variables: the crop density for

non-diseased susceptible (H_S) and partially resistant (H_R) cultivars and the pathogen density for the avirulent (A) and virulent (V) pathogens.

Throughout this paper, we only consider seed mixture (i.e. susceptible and resistant cultivars are both present in the same field, unlike in the case of patches) where the ratio θ of resistant cultivar is constant across seasons. Because of the discontinuity (seasonality) of the model, we introduced transition rules: the amount of pathogen at the start of one season is a fraction λ of the amount left at the end of the previous season. Within each season, we also assume that both strains of the pathogen have a death rate μ, which is constant across seasons. Also, we define a "coefficient of partial resistance" p varying from 0 (completely resistant) to 1 (completely susceptible) and we assume that we have a pathogen base infection rate β.

The initial results of this project we are presenting in this paper were aimed at comparing different hypotheses and ways to approach the problem of durability of resistance in order to decide on the best approach to take according to the objectives we are trying to achieve. In earlier results (Pietravalle et al., 2004), we have already presented the different conclusions three definitions of "durability of resistance" lead to. The time until take-over of the virulent pathogen is defined as the time needed, after its introduction into the system, for the virulent pathogen to reach a given threshold in the pathogen population (i.e. the time until $V/(V+A) \geq x\,\%$).

Other possible definitions are the "estimated green leaf area duration (yield) gain due to the use of the resistant cultivar" (where the green leaf area duration is calculated as the area under the density of non-diseased cultivars curve) and the "time of usefulness of the resistant cultivar" (i.e. the number of seasons needed before the estimated green leaf area duration gain per plant becomes negligible). However, in this paper, we only concentrate on the time until take-over of the virulent pathogen.

First, we compare simulations assuming monocyclic and polycyclic diseases. We have one main difference in the model in these two cases: we used a fixed ascospore arrival function (Gaussian, quadratic, etc.) at the start of the season for the monocyclic disease whereas we use a number g of spores produced per infection (i.e. per cycle) for the polycyclic disease. Also, even if the transfer rate parameter λ remains the same in both cases, it is important to note that its interpretation does differ slightly. For polycyclic diseases, it is the proportion of pathogen left over through the crop-free season via infected material whereas for monocyclic diseases, it represents the effect of environmental conditions through the crop-free period on the scale of the ascospore arrival function. When comparing those two cases, we restricted ourselves to one definition of partial resistance. In this definition, we assume that the avirulent pathogen has a smaller infection rate $p.\beta$ on the partially resistant cultivar. This leads to the following set of equations.

$$\frac{dH_S}{dt} = r_S\, H_S\, (1-\frac{H_S}{K_S}) - \beta\, H_S\, (V+A)$$

$$\frac{dH_R}{dt} = r_R\, H_R\, (1-\frac{H_R}{K_R}) - \beta\, H_R\, (V+p\,A)$$

$$\frac{dA}{dt} = g\,\beta\, A\,(H_S + p\,H_R) - \mu\, A$$

$$\frac{dV}{dt} = g\,\beta\, V\,(H_S + H_R) - \mu\, V$$

with: $K_R = \theta.K$, $K_S = (1-\theta).K$, $H_R(0) = \theta.H_0$, $H_S(0) = (1-\theta).H_0$

where: *V(0)* and *A(0)* are fixed the first season, then

V(0) = λ.V(end of previous season)

A(0) = λ.A(end of previous season)

where: r is the crop growth rate, K is the crop maximum density, H_0 is the crop sowing density and g is the number of spores produced per infection.

For polycyclic diseases, we introduce a second way to define partial resistance; in this case, the avirulent pathogen produces fewer spores ($p.g$) on the partially resistant cultivar. This leads to the following set of equations.

$$\frac{dH_S}{dt} = r_S\ H_S\ (1-\frac{H_S}{K_S}) - \beta\ H_S\ (V_S + V_R) - \beta\ H_S\ (A_S + p\ A_R)$$

$$\frac{dH_R}{dt} = r_R\ H_R\ (1-\frac{H_R}{K_R}) - \beta\ H_R\ (V_S + V_R) - \beta\ H_R\ (A_S + p\ A_R)$$

$$\frac{dA_S}{dt} = g\ \beta\ (A_S + p\ A_R)\ H_S - \mu\ A_S$$

$$\frac{dA_R}{dt} = g\ \beta\ (A_S + p\ A_R)\ H_R - \mu\ A_R$$

$$\frac{dV_S}{dt} = g\ \beta\ (V_S + V_R)\ H_S - \mu\ V_S$$

$$\frac{dV_R}{dt} = g\ \beta\ (V_S + V_R)\ H_R - \mu\ V_R$$

where: $\begin{matrix} A = A_S + A_R \\ V = V_S + V_R \end{matrix}$ where A_S is the density of the avirulent pathogen on the susceptible cultivar (similarly for A_R, V_S and V_R).

Results and discussion

Figure 1 compares the time until take-over as a function of the proportion of resistant cultivar θ for different pathogen death rates μ. This indicates that the less resistant cultivar there is present in the field, the longer it takes for the resistance to be broken down. Given this measure of durability of resistance, this result is easily explained by an effect of dilution, that is the less resistant cultivar available for infection, the smaller the virulent pathogen advantage over the avirulent. This also proves one limitation of this definition. Indeed, although the virulent pathogen does not invade very rapidly when the resistant cultivar is scarce, the benefits of using a resistant cultivar (with regard to yield for instance) are then very limited as well and this latter factor is not taken into consideration by that definition. Comparing figures 1a and 1b shows a very similar trend in the results although, because of a different choice of pathogen death rates, the values obtained for the durability of resistance are not directly comparable. Although we do not show the results in this paper, we also looked at the relationship between the transfer rate λ and the time until take-over and found that the time until take-over did not seem to depend on the transfer rate λ. However, similarly to what was found for the proportion of resistant cultivar θ, similar results were found for the monocyclic and polycyclic diseases. If similar results proved correct in further cases, this would make it possible to consider the simpler polycyclic model as our base model for more detailed studies instead of the monocyclic one.

The second set of comparisons made investigate the two possible ways to define partial resistance for polycyclic diseases. Figure 2 shows one such comparison for the time until take-over as a function of the coefficient of partial resistance p for a range of infection rates β. Although, yet again in this case, figures 2a and 2b show similar trends, it is interesting to observe that the time until take-over is much shorter when we define the partial resistance as affecting the number of spores produced per infection. Also, and as already discussed previously (Pietravalle et al., 2004), this shows a second limitation of the time until take-over as a measure of durability of resistance. Indeed, if taken to the extreme, and once again because no account is made of any possible gain (in yield for instance), this shows that "susceptible is durably susceptible".

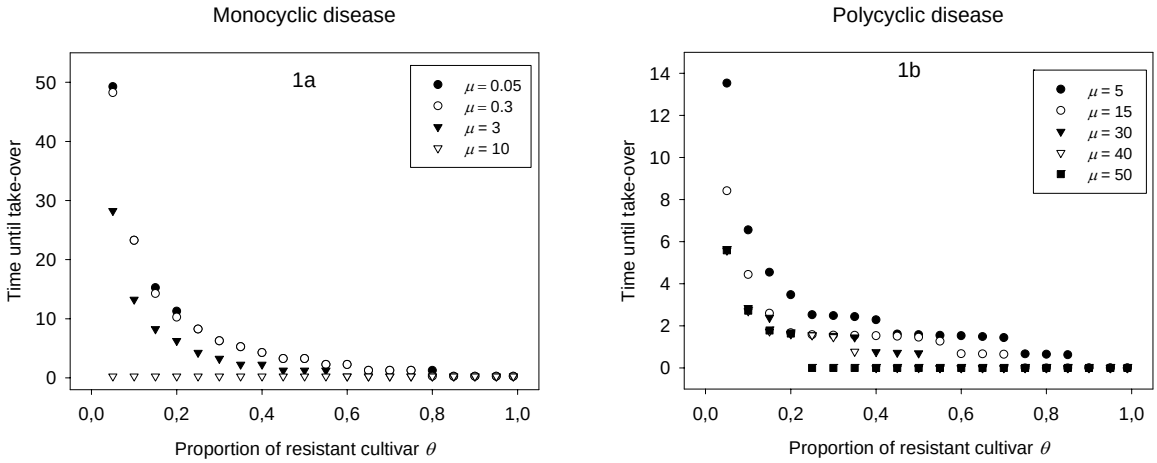

Figure 1. Comparison of the time until take-over as a function of the proportion of resistant cultivar θ for several pathogen death rates μ assuming (a) a monocyclic disease and (b) a polycyclic disease.

In conclusion, the aim of this work has been twofold. First, it has allowed us to emphasize the importance of accurately defining "durability of resistance". Second, as demonstrated in this paper, it has allowed us to investigate the effects of the model complexity on the output of the simulation. As a result of these findings, we can now envisage looking at further extensions of our model, in order to compare and advise with regard to different farming practices to optimize durability of resistance, after reverting back to a simpler model. Next steps will therefore be (1) the introduction of fitness cost through variable pathogen infection rates β, (2) the introduction and study of the effects of spatial deployment on durability of resistance (i.e. from seed mixture to separate plots), (3) the introduction and study of the effects of rotational use of resistant cultivars on durability of resistance and (4) the introduction and study of the effects of major genes pyramiding on durability of resistance.

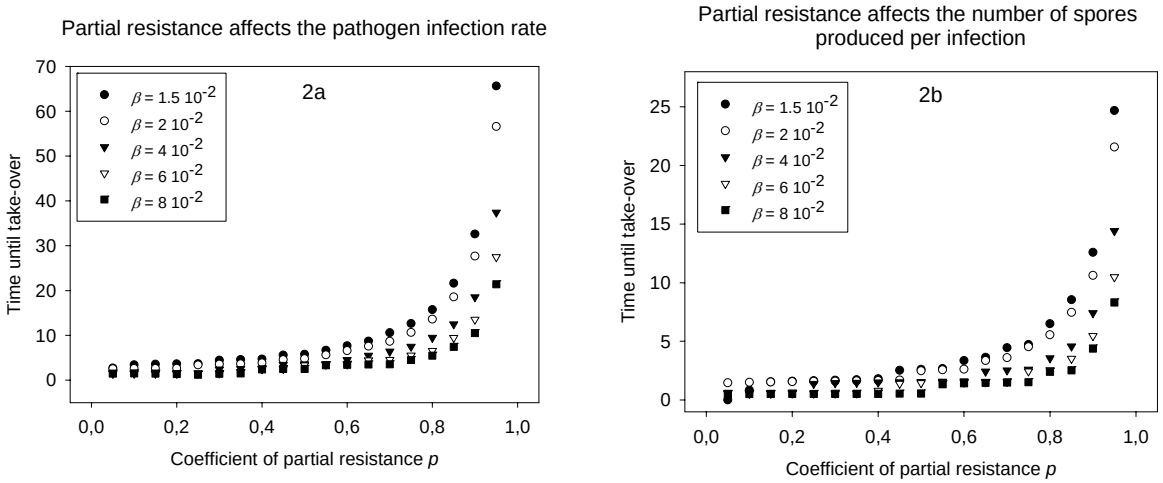

Figure 2. Comparison of the time until take-over as a function of the coefficient of partial resistance p for several pathogen infection rates β when partial resistance affects (a) the pathogen infection rate β and (b) the number g of spores produced per infection.

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LeptoNet and SPEC - new projects supporting the control of stem canker of oilseed rape in Poland

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Abstract: Phoma leaf spotting, upper stem lesions and stem canker, caused by the ascomycete species complex *Leptosphaeria maculans* and *L. biglobosa* has been reported in Poland since late 1950s. For most of this time the less aggressive *L. biglobosa* was prevailent, but recently the more aggressive *L. maculans* has started to be more common. Lepto-net and SPEC are new projects designet to support the control of the disease in Poland. Lepto-net is a system of several fields of oilseed rape with cultivars with no-known resistance genes. These will be sampled in order to collect a high diversity of *Leptosphaeria* spp. isolates from infected plants and a subsequent study of pathogenicity groups and races of the fungus. SPEC (the Polish abbreviation of the System of Forecasting of Disease Epidemics) is a network of six spore traps located in five different regions of the most intensive cultivation of oilseed rape in Poland. The traps will be monitored for ascospore release and the information will help determine the most appropriate time to apply fungicide spray in each region. The results will be available for farmers, extension services, breeders, researchers and commercial services through non-profit web sites of the Institute of Plant Genetics (www.igr.poznan.pl) and DuPont-Poland (www.dupont.pol.com).

Key words: spore trap, molecular detection, *Leptosphaeria maculans, Phoma lingam*

Introduction

Stem canker of oilseed rape has been observed in Poland since late 1950s, but the first outbreak of the disease was reported in north-west Poland in mid-1980s (Frencel *et al.* 1991). However, the disease is now pandemic across the whole of Poland. For several years the phoma leaf spot symptoms on leaves were not readily observed. Usually the first visible symptoms appeared on stems in early to mid-June at the green pod growth stage. These early symptoms developed very fast and sometimes the plants were killed before harvest. A survey of the population of *Leptosphaeria maculans* in Poland showed that the majority of fungal strains isolated from infected tissues belonged to the less aggressive type of the pathogen then called B-type or Siro^0, now identified as *Leptosphaeria biglobosa* (Shoemaker & Brun 2001). Between 1984 and 1996, only 5 % of the isolates represented the highly aggressive type of *L. maculans* (A-type or Siro$^+$) (Jedryczka *et al*. 1997). The situation in Poland contrasted with the situation observed in western Europe where most of isolates belonged to the more aggressive *L. maculans* (Jedryczka *et al.* 1999b). However, since the mid-1990s, symptoms on leaves and plants at the flowering stage were observed more frequently and this coincided with an increased number of isolations of the aggressive type *L. maculans* (Jedryczka *et al.* 1999a, Karolewski *et al*. 2002). The development of a generative stage of the fungus greatly depends on weather conditions (Toscano-Underwood *et al.* 2000), which makes it possible to develop decision support systems (Salam *et al.* 2003).

Two new initiatives have been recently undertaken to support the control of stem canker in Poland, the first should facilitate the characterization of the fungus population and the

second should allow the study of ascospore release of *Leptosphaeria* spp. in different regions of the country. The paper presents the general and specific aims of both projects, the methods to be used and inferences as to the best way to utilise the expected results.

Materials and methods

What is Lepto-net?

Lepto-net is designed to help to develop the system supporting the decisions on choice of rapeseed cultivars resistant to pathogenicity groups/races of *L. maculans* present in different regions of Poland. The project which began in autumn 2003 is a network of fields with "trapping" cultivars. The spring oilseed rape cv. Drakkar is known to lack known resistance genes to *L. maculans* (Rouxel *et al*. 2003), hence, under favourable conditions for plant infection, all ascospores should be able to invade plants and cause disease symptoms. However, the experiment performed for the EU FP5 project QLK5-CT-2002-01813 termed SECURE (Evans *et al*. 2003) showed that spring cultivars do not survive autumns and winters with low temperatures, that are often present in Poland. Therefore, Lepto-net uses also a winter cv. Darmor, which harbours one known resistance gene *Rlm9* (Delourme *et al*., 2004). The concept was based on the large-scale survey of race structure in France (Balesdent *et al.* 2004), followed by experiments performed for SECURE project. At present the Polish network comprises fields with spring cv. Drakkar and winter cv. Darmor, grown in 13 places in different regions (Figure 1). The system is supported by the Central Cultivar Testing Station (5 fields), the Institute of Plant Genetics PAS (2 fields), the Institute of Soil Science and Plant Cultivation (2 fields), the Agricultural University of Poznan (1 field), the Institute of Plant Protection (1 field) and two oilseed rape breeding stations of Plant Breeding Strzelce, located in Borowo and Malyszyn (1 field each). Leaves and stems of infected oilseed rape plants will be collected and studied for the composition of pathogenicity groups/races of *L. maculans* and their distribution among the regions of Poland.

What is SPEC?

The SPEC system, whose name originates from the Polish abbreviation of the System of Forecasting of Disease Epidemics (*pol*. System Prognozowania Epidemii Chorób) will be initiated in September 2004. It will comprise six spore traps located in the main oilseed rape growing areas of Poland, with five traps provided by DuPont-Poland in addition to the one operated by the IPG PAS since 1998 (West *et al.* 2002). The network of traps will allow the study of the release of *L. maculans* ascospores in different regions, encompassing Great Poland, Pomerania, Żuławy, Varmia, and Lower and Upper Silesia (Figure 2). In each region, a 7-day volumetric Burkard spore trap (Burkard Manufacturing Ltd., Rickmansworth, UK) (Figure 3) will be used. In Great Poland, an additional multi-vial cyclone spore sampler will allow us to perform a molecular detection of ascospores directly from the particles collected from air samples (Calderon *et al.* 2002). Sampling sites are equipped with weather stations, allowing the collection of meteorological data which will allow us to relate the development of the generative stage of the fungus with temperature, rainfall, humidity, windspeed, length of snow cover and other information. Fungicide spray experiments, designed to relate symptom development and yield loss to the spray timing and ascospore release are also planned.

The coordination of both systems, the collection and isolation of fungal cultures, the recognition of pathogenicity groups and races of the pathogen and the investigation of the maturation of pseudothecia and ascospores of *L. maculans* and *L. biglobosa* in Poland will be performed at the IPG PAS with financial support from DuPont.

Seasons from 2003/2004 to 2005/2006

Research institutes supporting the project

1 COBORU

2 IGR PAN

3 HR Strzelce+ IHAR

4 AU Poznań

5 IOR

6 IUNG

Figure 1. The location of fields with trapping cultivars of oilseed rape in Poland (Lepto-net)

Seasons from autumn 2004 to autumn 2008

Regions in Poland and types of spore traps

1 Great Poland *V*

2 West Pomerania *V*

3 Żuławy+Varmia *V*

4 Lower Silesia *V*

5 Upper Silesia *V*

6 Great Poland *C*

V- volumetric C - cyclone

Figure 2. The location of spore traps in Poland

Figure 3. Types of spore traps used in the SPEC project: left: seven-day volumetric spore trap, right: multi-vial cyclone spore sampler (Burkard Manufacturing Inc.)

Expected results

Results expected from Lepto-net

Experiments performed within Lepto-net will allow: 1) an evaluation of the intensity of phoma leaf spotting and symptoms on stems in subsequent years and in different regions of Poland; 2) to monitor the ratio of *Leptosphaeria maculans* and *L. biglobosa* isolates within the pathogen population; 3) to study the geographical distribution of pathogenicity groups and races of *L. maculans* in Poland. The results will help provide advice on the selection of cultivars resistant to races of the pathogen prevalent in particular regions of our country.

Results expected from SPEC

Experiments performed within SPEC will allow: 1) a constant monitoring of pseudothecia maturation and ascospore release of *L. maculans* and *L. biglobosa* in five different regions of intensive cultivation of oilseed rape in Poland; 2) to study the effect of weather on development of pseudothecial maturation and ascospores of *L. maculans* and *L. biglobosa;* 3) to determine the optimal time for fungicide application for the protection of oilseed rape against stem canker; 4) to relate yield response and disease incidence and severity with spray timing and ascospore release.

More than 2000 farmers and representatives of the agricultural industry were informed about the benefits of Lepto-net and SPEC at conferences organised by DuPont and IPG within the EU project PAGEN (Naganowska and Bialous 2003) during the autumn 2003 and winter 2004. The addresses of all parties interested in the projects are recorded in a database which will allow the projects to disseminate information direct to the target audience. The results will be available to farmers and all representatives of the plant protection services in Poland, through non-profit web sites at the Institute of Plant Genetics (www.igr.poznan.pl) and DuPont-Poland (www.dupont.pol.com). The current data on ascospore release will be also provided through emails and SMS text messages directly to farmers and farm advisors.

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Development of an 'ascospore shower' method for inoculating oilseed rape leaves with *Leptosphaeria maculans*

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Abstract: Two types of inoculum (ascospores and conidia) and four inoculation methods (spore suspension droplet on unwounded leaf, spore suspension droplet on wounded sites on leaf, spore suspension sprayed onto leaves and dry ascospores 'raining down' onto leaves) were compared, to develop an efficient method for inoculation with *L. maculans* when inoculum is limited. Ascospores were more infective than conidia. Leaves on all plants inoculated with ascospores, whether ascospores were produced under natural conditions or from defined crosses between two compatible isolates, developed phoma leaf spots, regardless of the inoculation method. Leaves inoculated with conidial suspensions by droplet inoculation on wounded sites and by spraying developed lesions, while leaves inoculated by droplet inoculation without wounding produced no lesions. For droplet inoculation, the infection efficiency (% inoculated sites which produced lesions) of ascospores was 100% while the infection efficiency of conidia was 56.1%. With spray inoculation, one lesion developed for every nine ascospores used or for every 2.4×10^6 conidia used. For inoculation with ascospores produced by *in vitro* crosses, three times as many pseudothecia were needed to inoculate the same number of plants using an ascospore suspension as using the 'ascospore shower' method. Since it is difficult to obtain enough ascospores from crosses to make ascospore suspensions, the 'ascospore shower' was chosen to study the fitness of near isogenic isolates of *L. maculans*.

Key words: pseudothecia, ascospores, conidia, infection, stem canker/blackleg

Introduction

Leptosphaeria maculans is an important pathogen of cruciferous crops, especially *Brassica* species, worldwide, causing phoma stem base canker (blackleg) of oilseed rape in Europe, Canada and Australia (West et al., 2001). Although both ascospores and conidia are produced during phoma stem canker epidemics on oilseed rape, phoma stem canker is known to be a monocyclic disease, with epidemics initiated by air-borne ascospores (Gladders & Musa, 1980; West et al., 1999). In Europe, including the UK, there is no experimental evidence that conidia play a role in the development of stem canker epidemics (West et al., 2001). Although stem canker epidemics are initiated by ascospores, most previous controlled environment studies on *L. maculans* have been done with conidial inoculum (e.g. Hammond & Lewis, 1987; Johnson & Lewis, 1994) and it is only recently that experiments on leaf penetration and infection criteria have been done with ascospores (Toscano-Underwood et al., 2001; Huang et al., 2003). Additionally, to obtain lesions using conidial inoculum leaves have had to be wounded before inoculation. This methodology is not suitable for studying mechanisms of interactions between the pathogen and host, since wounding could induce plant defence responses. For comparing the fitness of near isogenic lines (NIL) of *L. maculans* (Attard et al., 2002) wounding the plants before inoculation could hide differences between NILs in components of fitness related to plant infection. Since the successful production of ascospores

by crossing the two opposite mating types *in vitro* (Gall et al., 1994; Balesdent et al., 2001), the use of ascospores from defined isolates as inoculum to study the fitness of virulent and avirulent isolates of *L. maculans* became possible. However, since numbers of ascospores produced *in vitro* are limited, the methods for inoculating with ascospores produced under natural conditions (in which many ascospores can be produced on infected debris) are not suitable for inoculating with ascospores from defined crosses. This paper describes the development of an 'ascospore shower' method and discusses the potential use of the method.

Materials and methods

Preparation of plant material and inoculum

Oilseed rape seeds, cv. Lipton, were sown in 7 cm diameter pots containing peat-based compost and a soluble fertiliser (1·5 kg PG mix m^{-3}; Petersfield Products, Cosby, Leicester, UK). Plants were grown in a glasshouse and thinned to one plant per pot 10 days after sowing. Pots were then placed in seed trays and transferred to a 15ºC controlled-environment cabinet. Plants were grown in the cabinet for another 14 days until each had two true leaves fully expanded, with the third leaf just starting to expand.

A conidial suspension of *L. maculans* was prepared from a 12-day-old culture of isolate L44, which was derived from a phoma leaf spot on an oilseed rape leaf from a 2001/02 field experiment at Rothamsted. An ascospore suspension of *L. maculans* was prepared from naturally infected UK oilseed rape stem base debris collected in August 2002 using the method described by Huang et al. (2001). Ascospores from defined crosses were obtained by crossing *L. maculans* isolates V23.1.2 and V23.1.3 (Balesdent et al., 2001) on V8 juice agar using the method described by Gall et al. (1994). To make ascospore suspension using ascospores produced *in vitro*, pieces of agar with mature pseudothecia were attached to the inside of a Petri dish lid, then the lid was placed over the dish base. The dish was placed at 15°C for 6-8 h to allow ascospores to discharge into the bottom of the dish. The dish was observed under a microscope to confirm that ascospores were discharged. Distilled water was added to make an ascospore suspension. The concentration of conidia was adjusted to 10^6 conidia per ml and the concentration of ascospores was adjusted to 10^3 ascospores per ml, using a haemocytometer.

Inoculation

For inoculation with conidial suspension, three inoculation methods were used; spray the whole plant with conidial suspension, pin-point inoculation with a 10µl drop of conidial suspension on the leaf surface without wounding the leaf, and pin-point inoculation with a 10µl drop of conidial suspension on a wounded site on the leaf surface. Three plants were inoculated by each inoculation method. For pin-point inoculation, only the first and second leaves of each plant were inoculated and 6-8 wounded sites or 10-12 unwounded sites on each plant were inoculated.

For inoculation with ascospores, three inoculation methods were used; spray the whole plant with ascospore suspension, pin-point inoculation with a 10µl drop of ascospore suspension on the leaf surface without wounding the leaf and 'rain down' ascospores onto the plant. To let ascospores 'rain down' onto plants, three small pieces (0.5cm × 2cm) of oilseed rape debris with mature pseudothecia were attached to the inside of a tray cover and the debris was sprayed with distilled water until run off. Alternatively, for defined crosses, pieces of agar with mature pseudothecia produced *in vitro* were attached to the inside of a tray cover. For both sources of inoculum, the tray cover was then placed over the tray with plants

(Fig. 1). Three plants were inoculated by each inoculation method. For pin-point inoculation, only the first and second leaves of each plant were inoculated and 4-6 unwounded sites on each plant were inoculated. For spray and pin-point inoculation, plants were immediately covered with polyethylene bags sprayed inside with distilled water to maintain a high relative humidity for 72 h. With ascospore shower inoculation, the duration of the ascospore shower was 2 h for ascospores from natural conditions and 24 h for ascospores from crosses *in vitro*; after the inoculation period, plants were sprayed with distilled water and then covered with polyethylene bags for 72 h to maintain a high relative humidity.

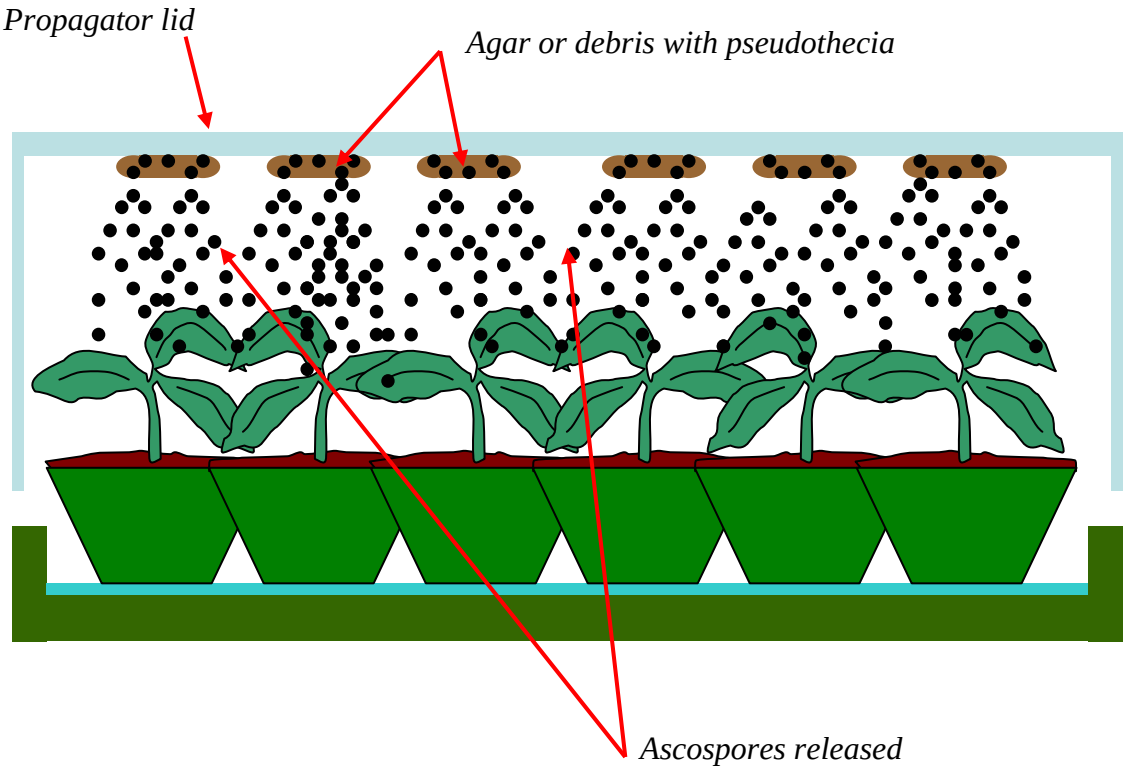

Figure1. Inoculation of oilseed rape seedlings with *Leptosphaeria maculans* ascospores produced under natural conditions on oilseed rape debris or *in vitro* by crossing the two opposite mating types on agar medium, using the ascospore 'shower' method.

To estimate the infection efficiency of conidia and ascospores inoculated by spraying, at the time of each inoculation three microscope slides were placed among the plants so that they were sprayed with conidia or ascospore suspension used for the experiment. The number of conidia or ascospores deposited per cm^2 on each of the slides was counted. The areas of leaves of five additional uninoculated plants of the same age were measured. The mean number of conidia or ascospores deposited per unit leaf area was calculated. Theses numbers were later related to the mean number of lesions that developed on leaves of the inoculated plants.

Assessment of disease

The numbers of new phoma leaf spots on each leaf of the inoculated plants were counted daily until no more new leaf spots appeared. The incubation period was estimated as time (days) from inoculation to the appearance of first phoma leaf spots. The infection efficiency

was estimated as the percentage of inoculated sites which produced lesions (for pin-point inoculation) or number of spores required to cause one lesion (spray inoculation).

Table 1 Effects of inoculum (ascospores or conidia) of *L. maculans* and inoculation methods on incubation period, number of lesions and infection efficiency

Inoculation method		Incubation period (days) [d]	No. lesions per plant	Infection efficiency [f]
Conidia [a]	Pin-point	- [e]	0	0
	Pin-point and wounding	15	4.1	56.1
	Spray	18	0.6	2.4×10^6
Ascospores from natural conditions [b]	Pin-point	5	5.4	100
	Spray	7	45.5	8.7
	Ascospore shower	9	6.5	- [e]
Ascospores from defined crosses [c]	Pin-point	7	4	100
	Ascospore shower	9	3.5	- [e]

[a] The conidial suspension was made from an *L. maculans* isolate derived from a leaf lesion on a UK winter oilseed rape crop.
[b] Ascospores were obtained from naturally infected UK winter oilseed rape stem base debris.
[c] Ascospores were obtained from crosses *in vitro*.
[d] Time (days) from inoculation to appearance of the first lesion.
[e] Not available or not tested.
[f] For pin-point inoculation, infection efficiency is defined as the percentage of inoculated sites which developed lesions; for spray inoculation with spore suspension (conidia or ascospores), infection efficiency is defined as the number of spores required to cause one lesion.

Results

Development of lesions

All the plants inoculated with ascospores produced on oilseed rape stem base debris under natural conditions developed leaf lesions, regardless of the inoculation method (Table 1). However, no lesions developed on plants inoculated with conidial suspension by droplet inoculation without wounding, and very few lesions developed on plants inoculated with conidia by spraying. Plants inoculated with conidia on wounded sites developed lesions. When the plants were inoculated with ascospores produced *in vitro* by crossing, they all developed lesions. The incubation period (from inoculation to appearance of the first lesion) was longer for conidia (15-18 days) than for ascospores (5-9 days). For both conidia and ascospores, incubation periods differed between different inoculation methods. For ascospores, the incubation period was longer for the ascospore shower (9 days) inoculation

method than for droplet inoculation (5-7 days). For conidia, the incubation period was longer for spray inoculation (18 days) than for droplet inoculation (15 days).

Infection efficiency

The infection efficiency of ascospores was greater than that of conidia (Table 1). For droplet inoculation, only 56.1% of sites inoculated with conidia developed lesions, while 100% of sites inoculated with ascospores, either produced under natural conditions or from crosses *in vitro*, developed lesions. In treatments where leaves were sprayed with spore suspensions, about 9 ascospores were required to produce one lesion, while it needed about 2.4×10^6 conidia to produce one lesion. Compared with inoculation with ascospore suspensions, the ascospore shower inoculation was more efficient. To inoculate three plants with ascospores from crosses between V23.1.2 and V23.1.3, three pseudothecia were used to produce the ascospore shower and each plant developed an average of 3.5 lesions (Table 1). By contrast, 10 pseudothecia were used to make the ascospore suspension and only 200 µl of ascospore suspension (at the concentration required, 10^3 ascospores/ml) was made. This volume was not enough for spray inoculation, and could be used only for pin-point inoculation; each inoculated plant developed an average of 4 lesions. To inoculate three plants with ascospores produced under natural conditions, three small pieces of debris were used to produce the ascospore shower and plant each developed an average of 6.5 lesions, while ten pieces of debris were required to make an ascospore suspension for spray inoculation.

Discussion

These experiments confirmed that ascospores of *L. maculans* are more infective than conidia. All the plants inoculated with ascospores, whether the ascospores were produced under natural conditions or from defined crosses, developed phoma leaf spots, regardless of the inoculation method. Plants inoculated with conidial suspensions by droplet inoculation on wounded sites and by spraying developed lesions, while plants inoculated by droplet inoculation without wounding the leaf developed no lesions. The infection efficiency of ascospores was greater than that of conidia, regardless of the inoculation method, suggesting that air-borne ascospores play an important role in initiating stem canker epidemics in autumn. These results agree with those comparing infectivity of ascospores and conidia of *Pyrenopeziza brassicae* (light leaf spot of oilseed rape) (Gilles et al., 2001).

The results suggest that the ‘ascospore shower’ inoculation method is an efficient method for inoculation with *L. maculans* when ascospore inoculum is limited, such as for inoculation with ascospores from *in vitro* crosses. Three times as many pseudothecia were required to inoculate the same number of plants when ascospores were used in suspension as when using the ‘ascospore shower’ method. Furthermore, when using the ascospore ‘shower’ inoculation method, it is not necessary to wound leaves, which more accurately simulates what occurs in natural conditions. As stem canker epidemics are initiated by air-borne ascospores, this method will enable research to more accurately investigate the host-pathogen interactions between *Brassica* species and *L. maculans*. Since new sources of cultivar resistance to *L. maculans* usually break down in a few seasons, the ascospore ‘shower’ method can be used to study the fitness of virulent/avirulent isolates, as part of work to investigate strategies to increase the durability of novel resistance genes (Rouxel et al., 2003; Brun et al., 2001).

Acknowledgements

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Spatial aspects of *Leptosphaeria maculans* (phoma stem canker) epidemiology

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Abstract: Two datasets were examined to investigate spatial aspects of phoma stem canker (blackleg) epidemics. In one experiemt, data were collected using a "grid" sampling method to investigate the spatial distribution during the winter period of the growing season. The data indicated that distributions of % leaves with phoma leaf spotting fitted a binomial distribution and did not indicate any local aggregation of disease. To investigate auto-correlation between disease measurements and distance between samples, variograms were estimated with the data for both assessments. For the January assessment, the variogram did not indicate any spatial auto-correlation. The variogram for the December assessment showed a continuous increase in variance of disease measurements up to an inter-sampling distance of about 15 m. This suggested that more data on a finer grid are needed to make more general statements about spatial distribution of phoma leaf spotting.

A second dataset, collected using a "cluster" sampling technique using "microplots" was subsequently analysed to investigate the spatial dynamics of the epidemic in more detail. Data at several sampling dates indicated that the incidence of plants affected was significantly different from a binomial distribution, indicating over-dispersal and local aggregation of disease between near-neighbour plants. However, there were differences between similar sampling dates in the two seasons of the study and the pattern was not consistent, presumably because of leaf fall. The results from both studies suggest that further research is required before the spatial dynamics of epidemic development and the specific role of ascospores and pycnidiospores of *L. maculans* during the epidemic can be fully explained.

Key words: aggregation, ascospores, over-dispersal, pycnidiospores

Introduction

Phoma stem canker, caused by *Leptosphaeria maculans*, is the most important disease affecting the UK oilseed rape industry causing yield losses of up to £40 million/annum (Fitt *et al*., 1997). The disease is regarded as being monocyclic with air-borne ascospores producing phoma leaf spots in the autumn. Initial leaf infections are followed by systemic growth *via* leaf veins/petioles to the stem where stem canker lesions subsequently develop (West et al., 1999) (Fig. 1). The current understanding therefore does not provide a role for pycnidio-spores, which *L. maculans* produces in abundance. Rain-splashed asexual pycnidiospores have been implicated in the localised spread of disease in Australia (Barbetti 1976). Theory suggests that if the disease is monocyclic, as suggested by West *et al* (1999), the spatial pattern of disease would initially be random and remain random throughout the season (through random infection by ascospores and disease progressing only from initial ascospore initiated infections). If the disease is polycyclic and pycnidiospores play a role in epidemic development, initial ascospore infection should be random but aggregation would be expected later in the season, following rain-splash of pycnidiospores from infected leaves to cause localised lesions on near-neighbour plants.

Detailed study of the spatial distribution and development of the phoma leaf spot / stem canker epidemic should therefore allow inferences to be made about the role of ascospores and pycnidiospores in the initiation and subsequent development of phoma stem canker epidemics. This paper describes work to analyse the spatial patterns of phoma leaf spotting in winter oilseed rape crops.

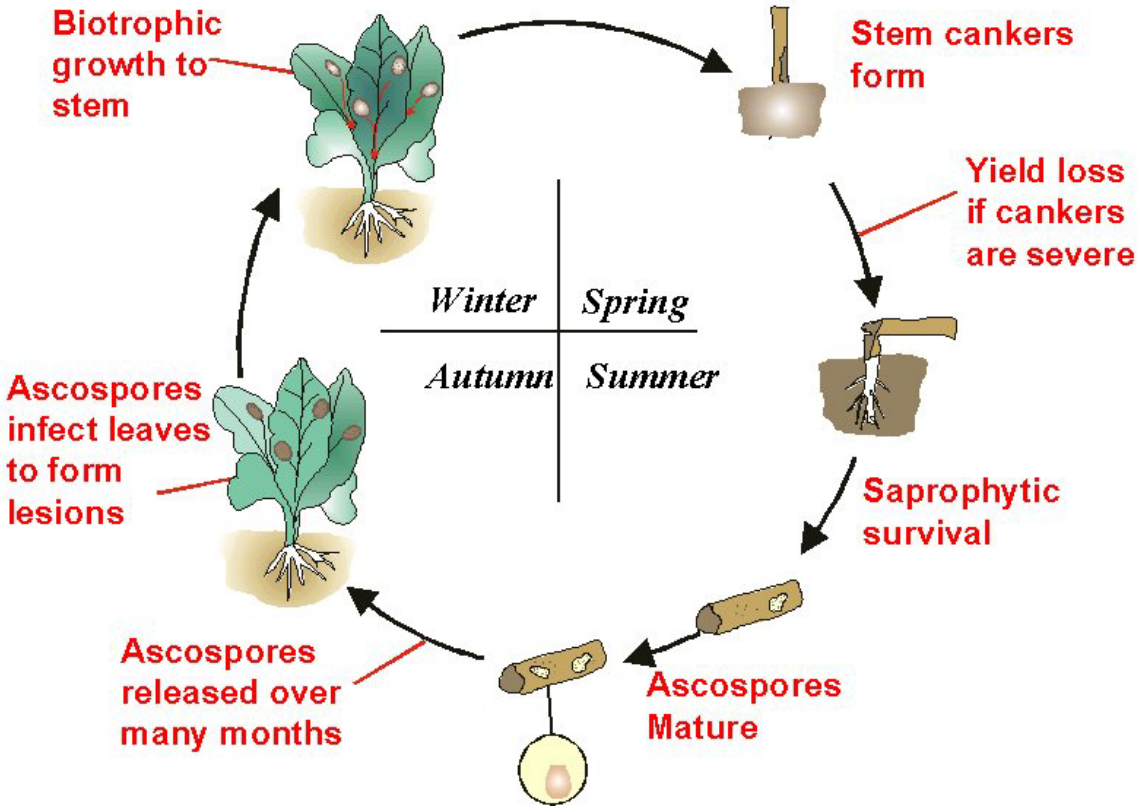

Figure 1. Monocyclic life cycle of *Leptosphaeria maculans* (adapted from West, *et al*., 1997).

Materials and methods

Dataset 1; Grid sample, 2002/03 season

Data for the first dataset were collected on the 18 December 2002 and 15 January 2003. The sample site was the area surrounding a fungicide timing experiment which, at the time the samples were taken, was untreated. The field was sown to oilseed rape cv. Apex (moderately susceptible with a resistance rating of 5). Sampling was done on a grid (72 sampling points with 8 x 8 m between adjacent sampling points). At each sampling point, four plants were assessed for the incidence of phoma leaf spots (% plants affected). In addition, to sample on a smaller scale, 60 plants were labelled (each at least 1 m apart) within three plots (10 m between plots) and assessed for phoma leaf spot incidence (% plants affected) at each sampling time interval.

Dataset 2: Cluster sampling, 1999/2000 season

During the 1999/2000 season an experiment was sown on the 27 August 1999 with cv. Apex. Eight sets of three microplots were set up adjacent to the untreated plots of a large inoculated

fungicide experiment (Fig.3). Ten plants per microplot (in an X configuration) were assessed for the number of phoma leaf spot lesions on each individual leaf every 2 weeks.

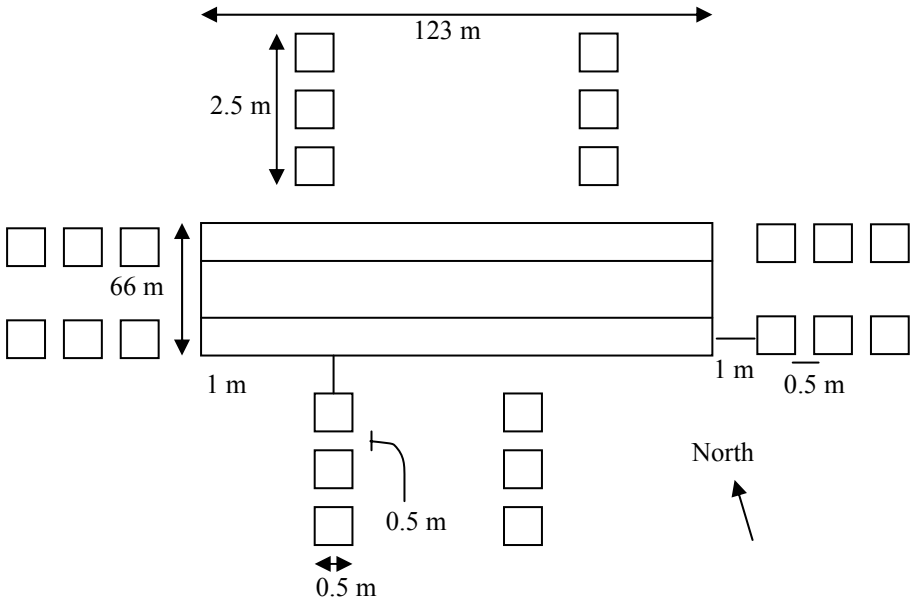

Fig. 3. Design of cluster sample experiment at Rothamsted 1999/2000 (cv. Apex). Microplots were set up adjacent to untreated plots within a large inoculated fungicide timing experiment. (Diagram not to scale).

Data analysis

Data were analysed using Genstat Version 5 (Payne *et al*., 1993). For Dataset 1, variograms based on 102 sample grid points (72 of the large 8m x 8m grid and 30 [means of pairs of tagged adjacent plants] of the small 1m x 1m grid) were estimated for each sample date. For Dataset 2, different distributions were fitted to the data to analyse the spatial aggregation pattern of the disease quantitatively. If infections were randomly distributed, mean disease incidence (proportion of plants affected) should be constant over all plots, and a binomial model can be used to describe the frequency distribution (number of plots) of incidence of phoma leaf spot (% plants affected). A generalized linear model with intercept term, logit link function and binomial error distribution was used to test the null hypothesis that infections were randomly distributed. The binary disease incidence data (plant infected, yes / no) were summed over the ten plants observed in each plot. Twice the scaled sum of the natural logarithms of the likelihoods (residual deviance) approximately follows a chi-squared distribution with degrees of freedom equal to number of plots minus one, under the null hypothesis. However, cycles of multiplication of a randomly distributed pathogen may cause differences in incidence between plots through aggregation and it was hypothesised that this additional spatial variation could be described by a beta distribution; this would produce a beta-binomial distribution to describe the observed pattern of disease incidence. Therefore, the log-likelihood was maximised as a non-linear function of the parameters. The likelihood that this particular number (out of 10) of infected plants occurred under the assumption of a beta-binomial distribution was derived for each plot.

Results and discussion

Dataset 1; Grid sample, 2002/3 season

Generally, on a large scale the level of disease was similar across the field at each sampling date. Patterns of dispersal followed a random pattern over the area sampled, although there were local effects (hotspots) within the field. The variogram for incidence of phoma leaf spotting on 18 December shows an increase in variation (decrease in correlation) between plants with increasing distance to each other (Fig. 3a). On 15 January, no increase in variation with distance was detected (Fig. 3b). This indicates that disease was aggregated within a range of approximately 5m at the earlier sampling date.

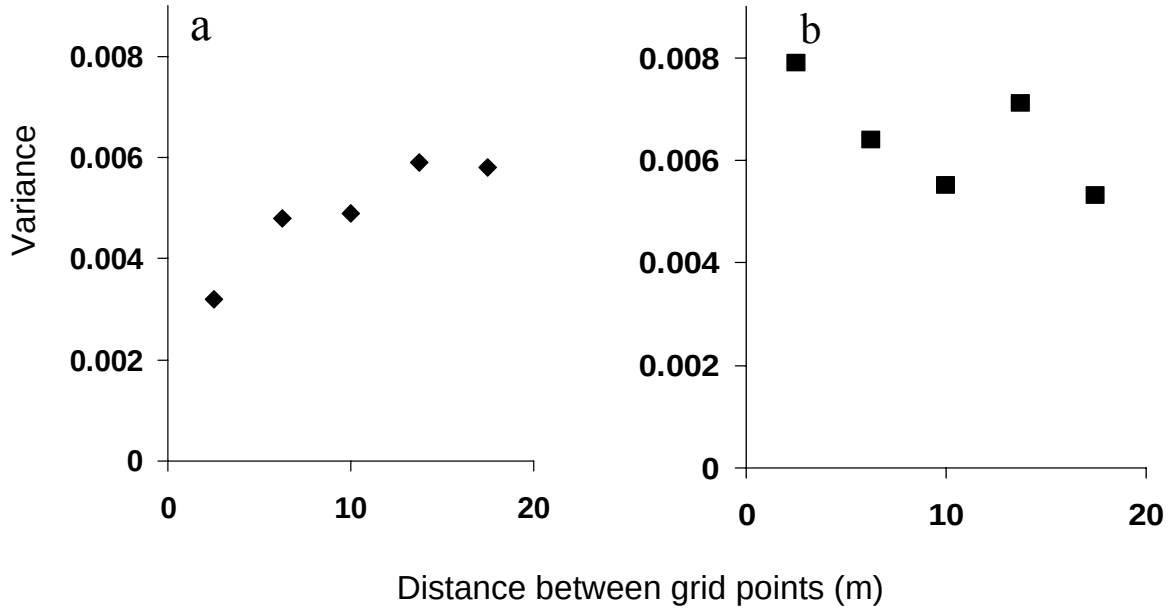

Figure 3. Variograms (indicating measures of variation) for two sampling dates; (a) 18 December 2002, (b) 15 January 2003 for oilseed rape phoma leaf spot incidence data collected from Bones Close field, Rothamsted, UK.

Data set 2; Cluster sampling, 1999/2000 season

In general, data appeared to be randomly distributed on a large scale as disease incidence was similar for each region of the field. However, comparing disease levels on microplots for individual sampling dates showed significant deviation from randomness. Disease incidence on 6 December 1999 (mean % plants affected = 81.8) showed larger variation than would be expected under the assumption of complete randomness. The deviance of the binomial model was 44.14 (with 23 d.f.) resulting in a dispersion parameter of 1.96, which was significantly greater than 1 (P = 0.005). Figure 4a indicates the deviation of the observed distribution of % plants affected from the binomial distribution which assumes randomness. The larger number of plots with 40 and 60 % plants affected and the low number of plots with 70 % plants affected are the main cause for the difference. A better fit was achieved with a beta-binomial distribution (Fig. 4a) which accounts for the increased variation. However, by the next sample date of 30 January 2000, the observed data was not significantly different from a normal distribution and was adequately explained by a binomial distribution (Fig. 4b). In this case, mean percentage of plants affected had increased to 89.8 %, however, the deviance of the binomial model was 27.1 (with 23 d.f.) resulting in a dispersion parameter of 1.18 which was not significantly different from 1.

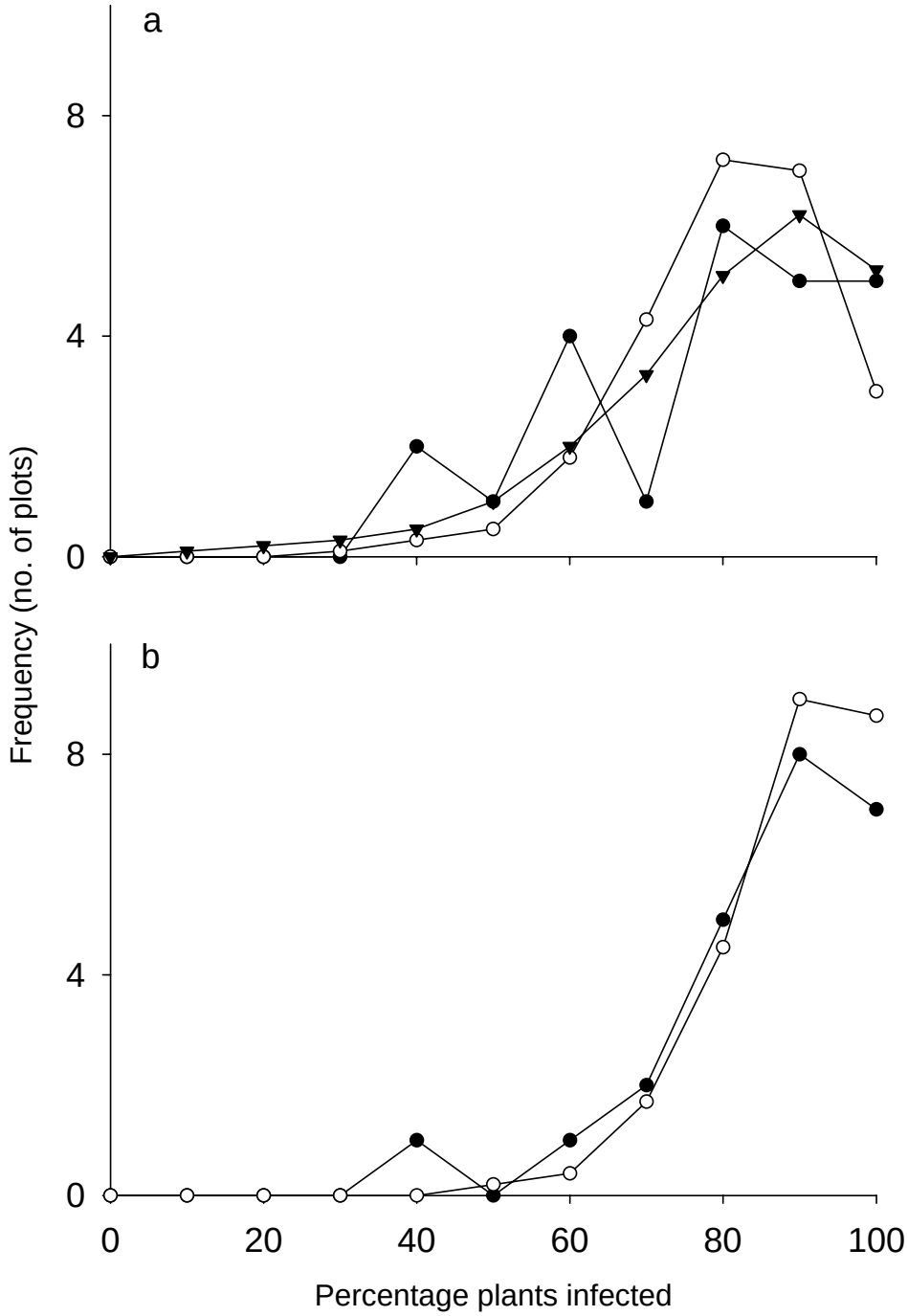

Figure 4. Fit of observed data (●) against predicted binomial distribution (○) and beta-binomial distribution (▼) for oilseed rape % plants affected with phoma leaf spot data collected on 6 December 1999 (a) and 30 January 2000 (b) from Great Knott II field, Rothamsted, UK.

In general, at most sample dates there was little evidence for aggregation within the data and the spatial distribution of phoma leaf spotting appeared to be random. However, when data from specific sample dates were analysed independently at certain points during the growing season, there was some evidence that the observed spatial pattern of the incidence of plants with phoma leaf spotting was non-random. Unfortunately, data were not collected later in the season to investigate whether this pattern was also observed in subsequent stem cankers. However,

Aubertot *et al*. (2004) recently investigated the spatial distribution of stem cankers at harvest and reported that although spatial distribution was generally random, significant spatial correlations were detected in some plots at the scales examined. They suggested that further studies were needed at a range of sampling scales before any conclusions could be made.

Aggregation would be consistent with localised secondary infections by rain-splashed pycnidiospores which develop after ascospore initiated primary infection. Although in Australia such a role has been suggested (Bokor *et al*., 1975) and demonstrated for pycnidiospores (Barbetti, 1976), current understanding of the life cycle, in Europe at least, suggests epidemics are monocyclic and that pycnidiospores play a very minor role in disease progression (West *et al.*, 2001). The current study suggests that the spatial aspects of the biology of the pathogen, and any implications which this might have on the relative importance of pycnidiospores within the life cycle, requires further experimental study.

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Effects of temperature and humidity on *Leptosphaeria maculans* symptom development on cotyledons of oilseed rape with different resistance genes

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Abstract: Artificial inoculation was performed on two cultivars and two lines of winter oilseed rape, bearing different specific resistance genes *Rlm2, Rlm3* and *Rlm9* originating from *Brassica napus* and *Rlm6* from *B. juncea*. The aim of the experiment was to study the effect of temperature and humidity on development of disease symptoms caused by the ascospores of *Leptosphaeria maculans*, a serious pathogen of oilseed rape worldwide. Two temperatures (15 °C and 20 °C) and three lengths (12, 24 and 48 h) of 100 % humidity following plant inoculation were used. The inoculation was performed using a drop of ascospore suspension on intact and wounded cotyledons and using an "ascospore shower" method. The results of study clearly demonstrated the efficiency of the resistance gene *Rlm6*. Other resistance genes did not protect plants from infection. Symptom development was faster and more severe with increasing temperature and lengths of wetness duration post inoculation. Differences in severity of symptom development between wounded and intact cotyledons reduced with increasing wetness duration.

Key words: stem canker, *Phoma lingam*, *Rlm6*, disease symptoms, effect of weather, resistance response;

Introduction

Infections of winter oilseed rape plants by *Leptosphaeria maculans* are initiated in the autumn by ascospores of the pathogen. Ascospores are released from mature pseudothecia - fruiting bodies of the sexual stage that form on contaminated stubble during the previous season. The rate of pseudothecia maturation depends on weather conditions, in particular on rainfall and relative humidity (West *et al*. 2002, Salam *et al.* 2003, Toscano-Underwood *et al*. 2003) and on soil moisture which affects stubble wetness (West *et al*. 2004). During wet summers, pseudothecia mature faster and release ascospores in early autumn. For example, in the autumn of 2003 in the region of Wielkopolska, central-west Poland, the first ascospores trapped by a Burkard trap were found on 9-12 September. At this time most plants of winter oilseed rape were at the cotyledon stage.

Temperature and wetness effect the development of disease symptoms on leaves (Biddulph *et al*. 1999, Toscano-Underwood *et al*. 2001) and stems (Sun *et al*. 2001). The experiments presented in this study were designed to show the effects of temperature and humidity on the development of disease symptoms on plants infected with ascospores of the pathogen, and the effect of resistance genes expressed in some new winter oilseed rape lines.

Materials and methods

Artificial inoculation was performed on two cultivars and two lines of winter oilseed rape bearing different specific *Rlm* resistance genes. The cultivar Darmor has one known resistance

gene *Rlm9* and polygenic resistance effective at the adult stage; the cv. Eurol has two known resistance genes *Rlm2 and Rlm3* (Pilet *et al.* 1998 and 2001, Rouxel *et al.* 2003, Delourme *et al*. 2004).

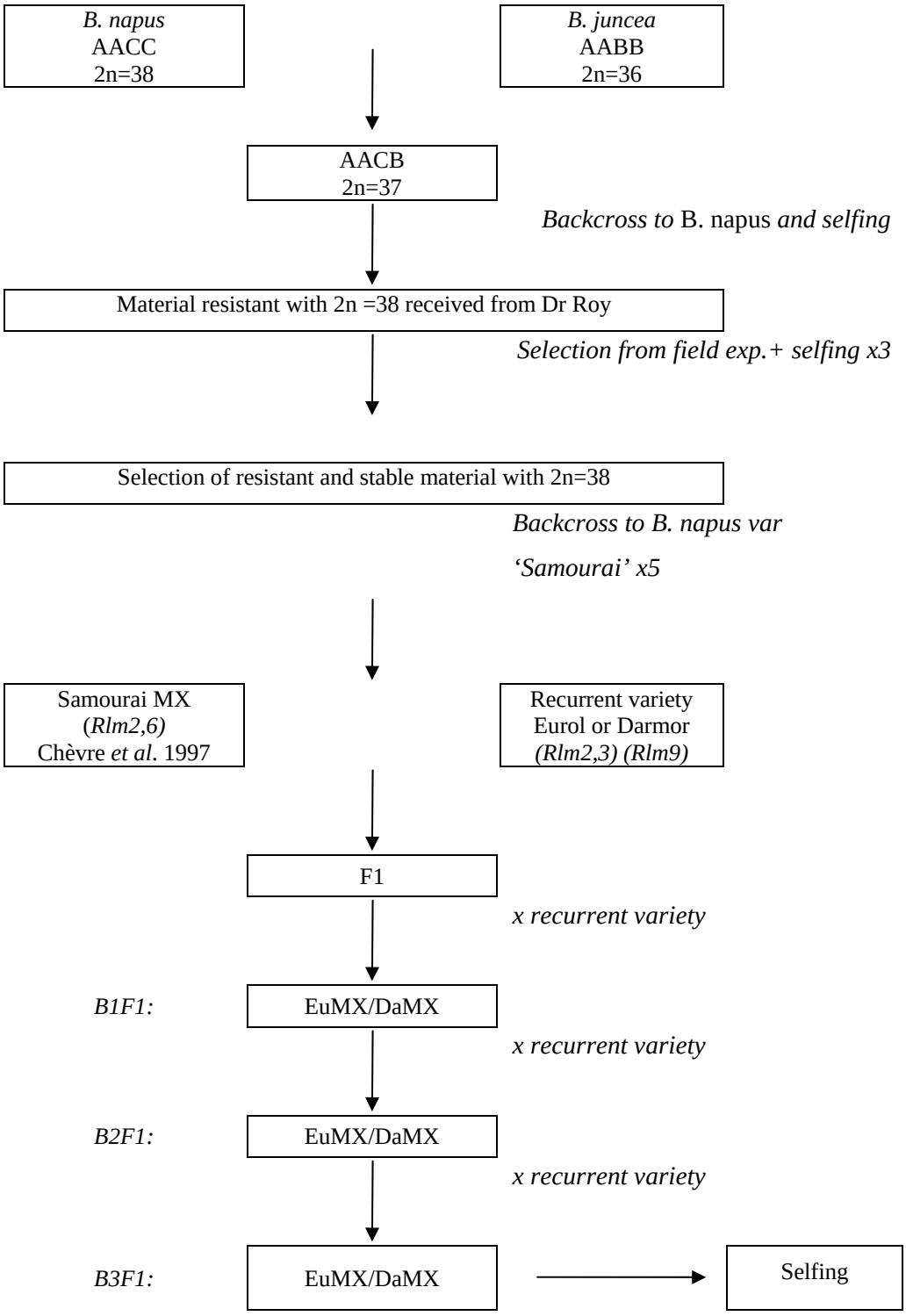

Figure 1. Scheme of the production of MX lines

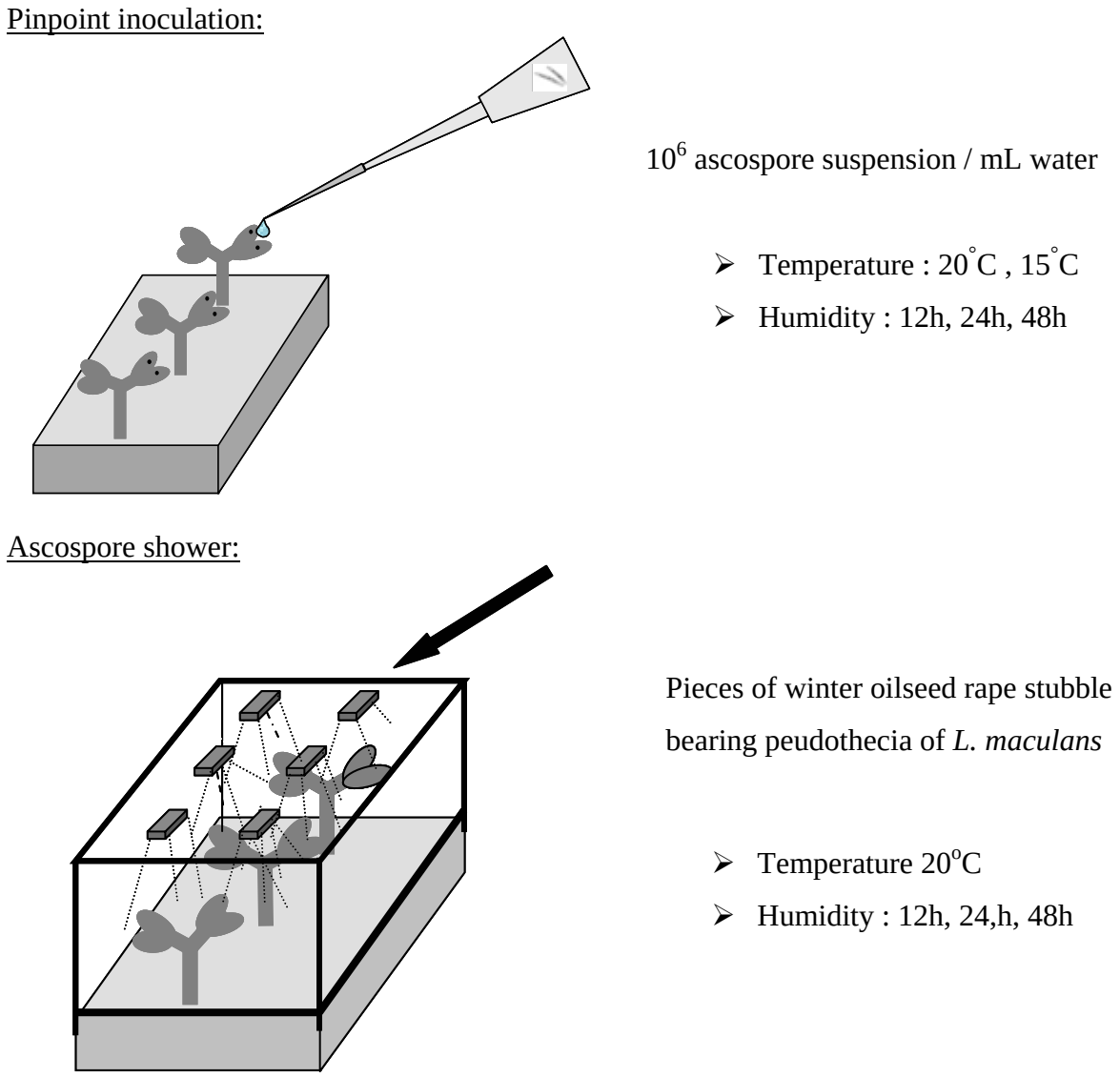

Figure 2. Comparison of pinpoint and ascospore shower methods of plant inoculation

Two lines named DarmorMX and EurolMX carry the resistance gene *Rlm6* introgressed into cvs. Darmor and Eurol from *Brassica juncea*. The initial resistant line carrying *Rlm6* (Chèvre *et al.* 1997) was back-crossed three times with the recurrent lines Darmor and Eurol. The introgression at the homozygous stage was obtained by selfing and by selection through cotyledon tests and/or molecular mapping (Barret *et al.* 1998). Three to seven selfing generations were performed for DarmorMX and EurolMX respectively. The production sequence of MX lines are presented in Figure 1.

Inoculation of plants was performed in controlled environment cabinets 12 days after sowing at 15 °C and 20 °C using two methods: 1) a suspension of ascospores of *L. maculans* at a concentration of 1 x 10^6 spores/mL; 2) an "ascospore shower" from fragments of winter oilseed rape stubble bearing pseudothecia of *L. maculans* (Huang *et al*. 2004). For the ascospore shower method of inoculation, six stem sections, each about 1.5 - 2.0 cm were used per 12 plants (1 replicate), 24 sections per 30 × 40 cm tray, to give one section per 50 cm^2. The comparison of both inoculation techniques is schematically presented in Figure 2.

Stubble used for the “ascospore shower” inoculations consisted of naturally infected stems harvested in 2003 at Rothamsted, UK. For both types of inoculation there were three different lengths of duration of 100% humidity after inoculation: 12, 24 and 48 h of full coverage of plants with plastic propagator lids, followed by 65% relative humidity for the rest of experiments. In order to ensure 100% humidity, the seed trays were filled with water and propagator lids were sprayed with water before they were placed on trays with inoculated plants. For inoculation with the ascospore suspension, two other factors were introduced: 1) intact cotyledons, 2) cotyledons wounded with a needle before the application of a 10 μL droplet of the ascospore suspension. Each treatment of the experiment (inoculation technique x temperature x lenght of 100% humidity) comprised 12 plants in 3 replicates, each in a different container (tray).

Plants were observed daily to determine symptom onset and speed of expansion. Phoma leaf spots resulting from the artificial inoculation were measured for all inoculated plants. Due to negligible variation between the replicates, the results presented in this paper are the mean values per treatment (36 plants).

Results and discussion

Effect of the new resistance gene Rlm6 from Brassica juncea

The results clearly demonstrated the efficiency of the introduced resistance gene *Rlm6* (Figure 3). In all treatments, the cultivars Eurol and Darmor were highly infected, whereas the cultivars with the additional gene *Rlm6* showed little necrotic symptoms. For *Rlm6* material, the average size of the largest symptom was a 4.9 mm necrotic spot, even in conditions conducive to disease development (20°C, 48 h high humidity after inoculation, 14 days post inoculation, wounded cotyledon of cv. DarmorMX). At 20°C, ascospore suspension inoculation of wounded cotyledons produced leaf spots ranging from 1.7 mm to 4.9 mm, depending on the length of the initial period of high humidity. For intact cotyledons the range of symptom size was from no symptoms to 3.05 mm. At lower temperatures (15°C) the spot sizes ranged from 1.2-3.2 mm or 0-2 mm for wounded and intact cotyledons, respectively. In the experiment inoculated using the ascospore shower at 20°C the average symptom size at the same time after the inoculation was lower and it ranged from 0 to 1.25 mm. However, this method achieved the same size of the largest symptom (average: 2 mm) two days later (on day 16). The *Rlm6* gene protected the plants for a longer time, at 20°C the symptoms on cultivars without this gene were observed on day 7, and with this gene they were noted 8-9 days post inoculation (dpi). The same effect of protection lasting one day longer was also observed at 15°C (8-9 or 9-10 dpi, respectively). This effect was more pronounced when plants were inoculated using the ascospore shower. In this case, symptoms on non-MX cultivars were observed on days 11-12 and on MX-lines they were observed on days 14-15. Only the 48 hour period of 100 % humidity after infection decreased this difference to 1-2 day delay. It must be stressed that even in case of the bigger symptoms, that were 3-5 mm diameter, the spots on cotyledons had a brown colour and a necrotic texture.

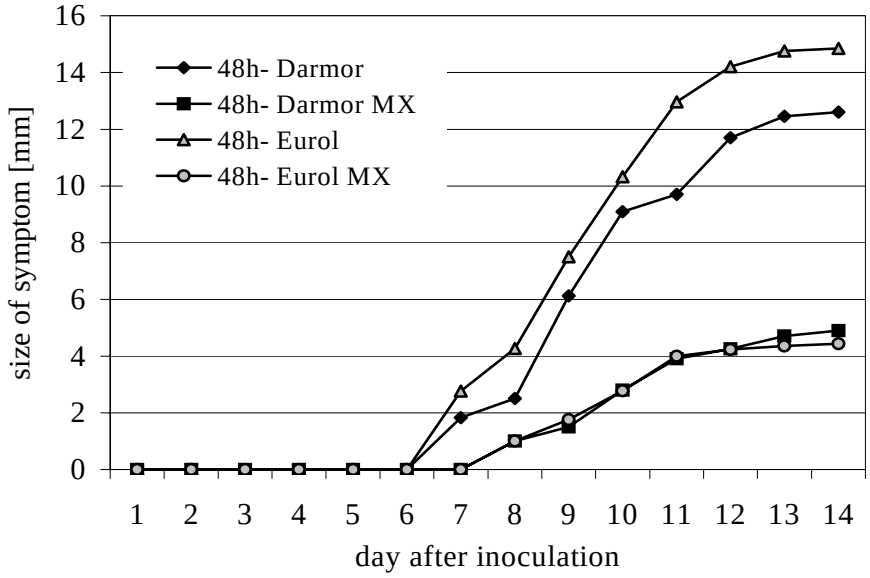

Figure 3. Effect of resistance gene *Rlm*6 on development of phoma leaf spots

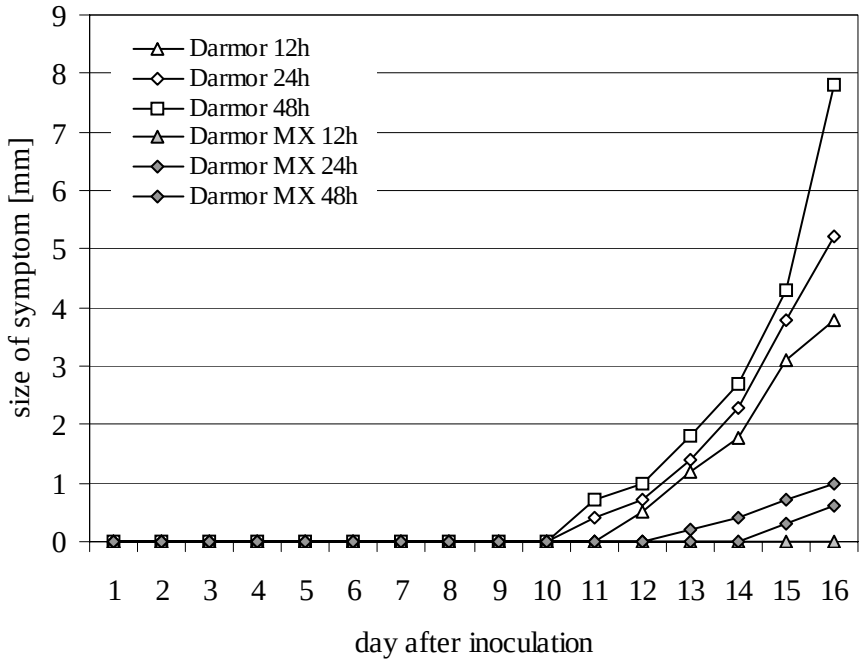

Figure 4. Comparison of phoma leaf spots on Darmor and Darmor MX line after cotyledon inoculation with the shower of *L. maculans* ascospores

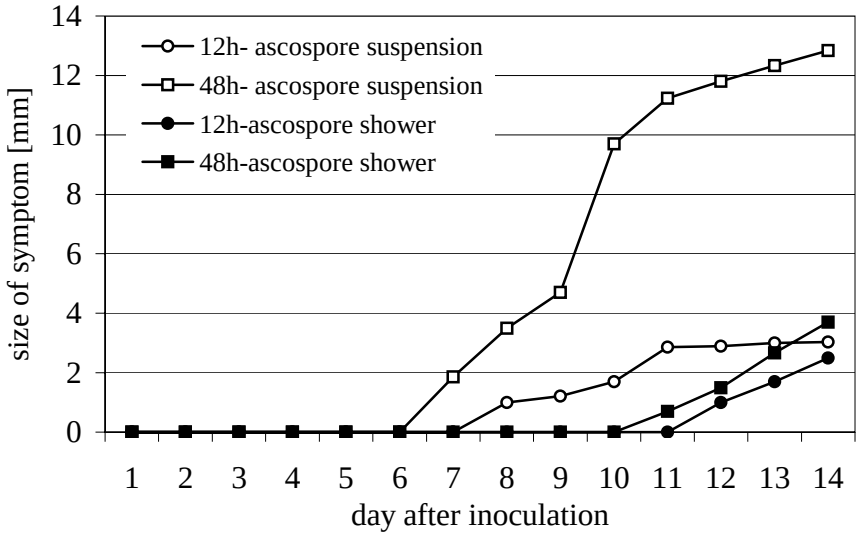

Figure 5. Development of disease symptoms on intact cotyledons of cv. Eurol inoculated with ascospore suspension and ascospore shower

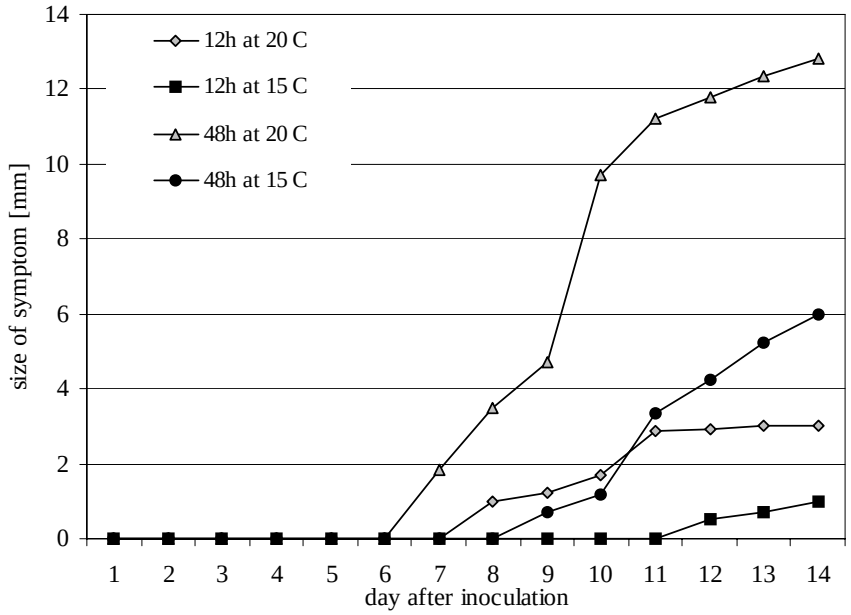

Figure 6. Effect of temperature on development of disease symptoms on intact cotyledons of cv. Eurol

***Effect of the resistance genes* Rlm*2,* Rlm*3 and* Rlm*9 from* Brassica napus**

The effect of the genetic background (*Rlm2, Rlm3* and *Rlm9* resistance genes from *Brassica napus*) is not as clear as the effect of *Rlm6* resistance gene introgressed from *Brassica juncea*. Artificial infection with the ascospore suspension at 20°C resulted in symptoms that were in all but one case slightly more severe for the cv. Darmor than for cv. Eurol, e.g. 14.85 mm

mean symptom diameter for Eurol and 16.37 mm for Darmor, 14 dpi, 20°C, 48 h high humidity, in treatments with wounded cotyledons. However, in other experimental treatments (ascospore suspension inoculation at 20°C and 15°C with intact cotyledons and also with the ascospore shower method) this rule was not observed. The *Rlm6* gene gave excellent resistance to the field population of *L. maculans* existing currently on the straw of oilseed rape from the UK. The other resistance genes did not produce a similar resistance response and were not able to prevent the plant infection. This result is in agreement to the very recent study of the population of *L. maculans* in the UK, showing that all studied isolates are virulent towards *Rlm2, 3* and *Rlm9* (Stachowiak *et al*. 2004).

Effect of the method of inoculation on disease symptoms

A comparison of the results obtained using two inoculation methods was possible for the 20 C inoculation treatment with intact cotyledons. In the case of inoculation with an ascospore suspension, the first results of inoculation were observed faster and they were more severe. Small initial symptoms on plants (*ca*. 1-2 mm diameter) were observed seven days after inoculation in treatments with 24 and 48 hours of high humidity after application of spore suspension, and eight dpi when the plastic lids were kept on for only 12 hours after the inoculation. Regarding the ascospore shower inoculation method, first symptoms were also observed for the treatments with 24 h and 48 h of high humidity, but four days later, on day 11. For the treatment where high humidity was kept for only 12 hours, the first symptoms were again observed with one day delay in comparison to the 24 h and 48 h humidity variants, that is 12 dpi (Figures 4 and 5). Infections on plants kept in high humidity for only 12 hours did not differ much in respect to lesion size in comparable period of time after inoculation. However, in 24 h and 48 h at 100% humidity the symptoms caused by 10^6 spores per mL of suspension were two or four times bigger in comparison to the symptoms caused by inoculation using ascospore shower. This is evidently caused by the higher concentration of ascospores in the suspension and the leaf wetness from the inoculation droplet allowing for fast germination and colonisation of cotyledons in comparison to the ascospore shower method, in which the initial humidity was very high, but the cotyledons were never wet.

Effect of temperature on development of phoma leaf spots

Development of disease symptoms occurred faster and more severely with increasing temperature (Figure 6). Initial symptoms were observed after 8 to 9 days after inoculation with the ascospore suspension at 15°C, when the cotyledons of the non-MX cultivars were wounded and 9 to 11 days when they were not wounded. At 20°C, initial leaf lesions were observed after 7 days (wounded cotyledons) or 8 days (intact cotyledons). The same rule applied to the MX lines, with one or two day delay.

Effect of humidity on development of phoma leaf spots

The propagator lids maintained small droplets of water through the whole period of cover, confirming 100% humidity, however the surface of cotyledons was dry. To study the effect of initial length of 100 % humidity, the plants were then kept at considerably lower humidity (65 %). Apart from the difference in concentration of the ascospores, that was higher in the method of inoculation with the ascospore suspension, the presence of drops of water on cotyledons treated with the suspension could be expected to explain the increasing speed of symptom development on cotyledons of oilseed rape. Similarly to the increased temperature, the symptom development was faster and more severe with increasing lengths of 100% humidity duration following inoculation (Figures 7 and 8). The symptoms observed in treatments with 12 h humidity always came out slower by one or two days in comparison to

the remaining treatments of 100% humidity (24 h and 48 h) and the symptoms observed two weeks after the inoculation were about three times smaller in case of inoculation of wounded cotyledons by the ascospore suspension at 20°C or even four (cv. Eurol) to six times (cv. Darmor) smaller when the cotyledons stayed intact. At 15°C the difference between humidity treatments was considerable: two (cv. Darmor) or three times (cv. Eurol) smaller symptoms at 12 h high humidity for wounded cotyledons and one-and-a-half (cv. Darmor) to six times (cv. Eurol) for the treatments with extreme lengths of humidity. For the ascospore shower the difference between the 12 h and 48 h treatments was similar for both cultivars (1.5 times smaller spots on cotyledons at 12 h). In case of 24 h and 48 h treatments of high humidity, the results were intermediate or the results for the 24 h treatment were closer to the 48 h treatment than to the 12 h treatment. With respect to MX lines, the high humidity also speeds the enlargement of spots on cotyledons, but they remain necrotic and below 5 mm diameter due to the presence of the *Rlm6* resistance gene.

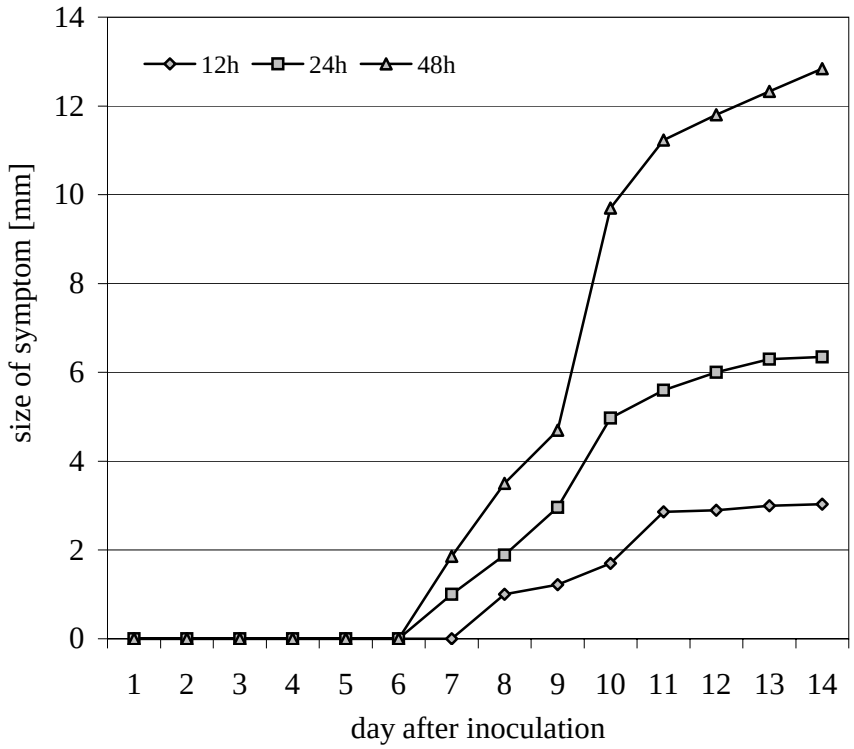

Figure 7. Effect of humidity on development of disease symptoms on intact cotyledons of cv. Eurol (*Rlm*2,3) at 20°C

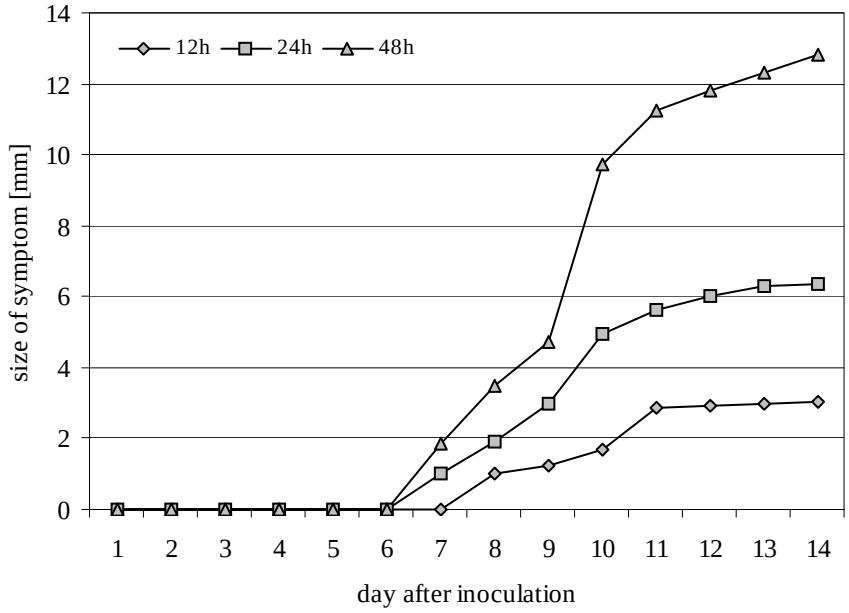

Figure 8. Effect of humidity on development of disease symptoms on intact cotyledons of cv. Darmor (*Rlm*9) at 20°C

Effect of wounding of the leaf tissue

Symptom severity was always greater on wounded cotyledons than on intact cotyledons inoculated with *L. maculans* ascospores (Figure 9). The difference was *ca.* 3 mm on average when comparing wounded and unwounded matching treatments, that is the same temperature, humidity and cultivar. Differences in severity of symptom development between wounded and unwounded cotyledons reduced with increasing wetness duration post inoculation.

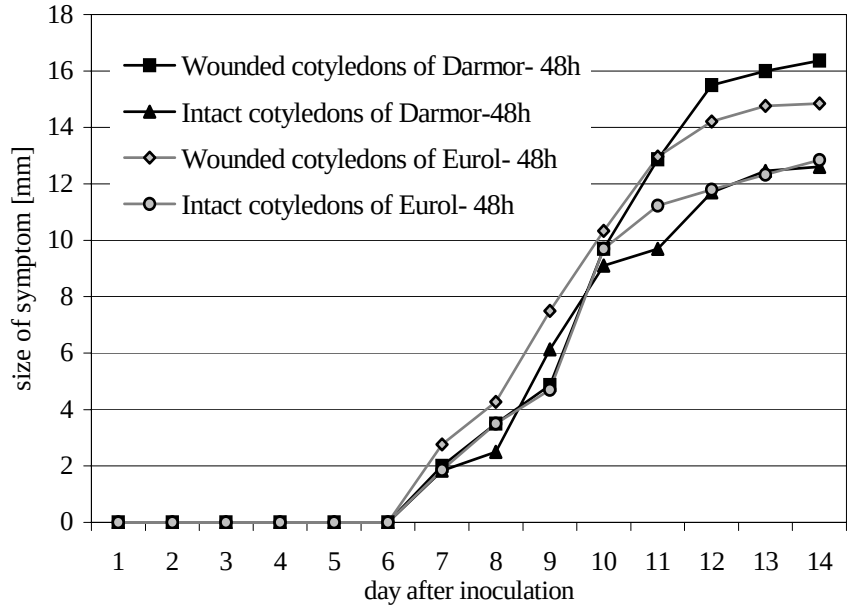

Figure 9. Effect of wounding on development of phoma leaf spots

Acknowledgements

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Leptosphaeria maculans, L. biglobosa and fungicides, preliminary results from *in vitro* and winter oilseed rape experiments

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Abstract: The sensitivity of *L maculans* and *L. biglobosa* to fungicides commonly used on UK winter oilseed rape was investigated *in vitro*. On medium containing flusilazole or tebuconazole, the radius of mycelial growth of isolates was recorded, and the effective dose at which 50% of growth was inhibited (ED_{50}) determined. Isolates of *L. maculans* had lower ED_{50} values, and the range of *L. maculans* ED_{50} values was narrower for both flusilazole (ED_{50} 0.27µg/ml, range 0.06-0.7µg/ml) and tebuconazole (ED_{50} 0.67µg/ml, range 0.4-0.94µg/ml) than for isolates of *L. biglobosa* (flusilazole ED_{50} 0.34µg/ml, range 0.16-0.9µg/ml; tebuconazole ED_{50} 1.45µg/ml, range 0.75-2.63µg/ml). The effects of fungicide on pathogen populations were investigated in a winter oilseed rape experiment in 2002/2003. The early fungicide treatment apparently affected pathogen populations, since fewer *L. biglobosa* isolates were detected on basal and upper stems from early treated plots than on stems from untreated and late treated plots.

Key words: phoma stem canker, disease control, population structure, fungicide resistance, triazole

Introduction

Over the past 20 years, the annual area of oil crops in the UK has increased by 400%, and at present around 450 000 ha of winter oilseed rape are cultivated each growing season (Anonymous 2003). Associated with this crop intensification, the occurrence of pests and diseases has increased. Since 1995 phoma stem canker, caused by a fungal species complex comprising at least two species (*Leptosphaeria maculans* and *L. biglobosa*, (Mendes-Pereira et al. 2003)), has been the dominant disease of oilseed rape in the UK, resulting in estimated losses of more than £30 M each year (Fitt et al. 1997). In Western Europe, control of this disease relies on the use of fungicides, as no oilseed rape cultivars with high levels of resistance are currently available.

In the UK, over 90% of winter oilseed rape crops receive one or more fungicide applications within a growing season. 696 300 ha of oilseed rape were treated with fungicides in 2003 (Anonymous 2004), each hectare of oilseed rape therefore receiving an average of 1.5 sprays. Triazole fungicides such as flusilazole and tebuconazole are the most commonly applied chemicals (Fig. 1) and, although resistance to methylbenzimidazole carbamate (MBC) fungicides has been demonstrated in other pathogens, carbendazim remains popular, being used in a single active-ingredient spray or in combination with flusilazole.

Despite the frequent use of fungicides, little is known about the effects these chemicals have on pathogen populations. We aim to investigate such effects and report first results.

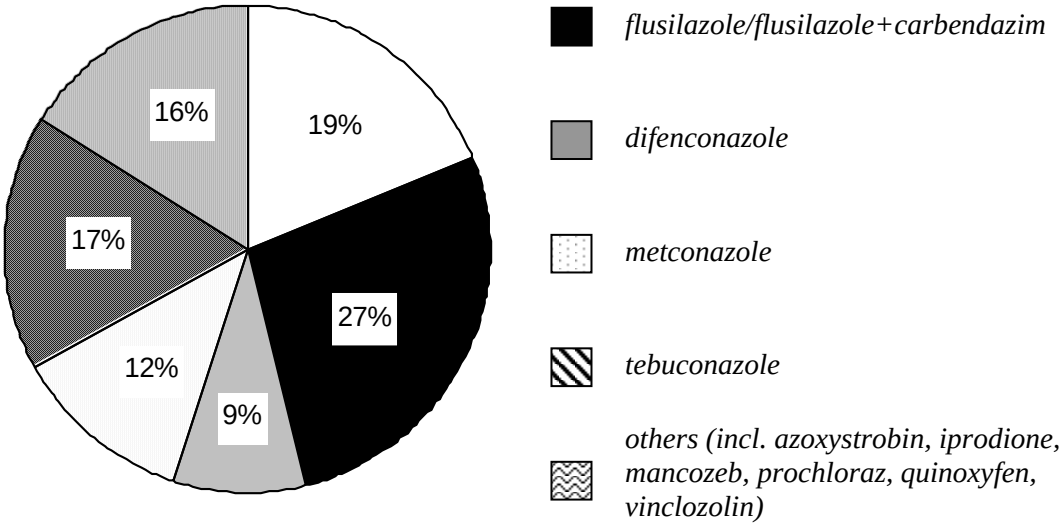

Figure 1. Fungicide usage on 2002-2003 UK winter oilseed rape crops (Anonymous 2004)

Materials and methods

Fungicide sensitivity of* L. maculans *and* L. biglobosa *isolates from England and Wales

Oilseed rape stems were collected in July 2003, at different sites in the UK (Fig. 2). 60 isolates of *Leptosphaeria maculans* and 60 isolates of *L. biglobosa* (Table 1) were obtained from stem base cankers or lesions on upper parts (>10 cm from stem base) of oilseed rape stems, using methods described in (West et al. 2002). These 120 hyphal tip isolates were identified *in vitro* according to colony morphology and pigment production (Williams and Fitt 1999) and 55 of the isolates were also identified by PCR.

In vitro assays were done in 24-well flat bottom microplates (Iwaki, Japan) with a well diameter of 16mm. Technical grade flusilazole (DuPont, UK) and tebuconazole (LGC Promochem Ltd., UK) were dissolved in 70% ethanol, to give stock solutions of 4 mg/ml. Potato Dextrose Agar (PDA) medium was prepared according to manufacturer's instructions (Oxoid, UK) and cooled to 47°C before fungicides were added. An initial experiment done to investigate the fungicide concentration suitable for a large-scale screen showed a difference in sensitivity between *L. maculans* and *L. biglobosa*. Isolates were therefore screened at adjusted ranges: 0, 0.05, 0.1 and 1µg/ml of flusilazole and 0, 0.5, 0.75 and 1µg/ml of tebuconazole for *L. maculans* or 0, 0.1, 0.5 and 2µg/ml of flusilazole and 0, 1, 2 and 4µg/ml of tebuconazole for *L. biglobosa*. Using sterile Pasteur pipettes, hyphal plugs were taken from the edge of actively growing cultures and inoculated into the centre of each well. Plates were sealed and kept at 15°C in darkness. Radial growth was assessed when the control culture (without fungicide) reached the edge of the well, after 7 days or 17 days of incubation for *L. biglobosa* or *L. maculans*, respectively. Three measurements per well were averaged and the value used to calculate the effective dose at which 50% of growth was inhibited (ED_{50} value). Data analysis was done with the statistical software package GenStat v6.2 (Payne et al. 1993). All assays were duplicated and the experiment was done twice.

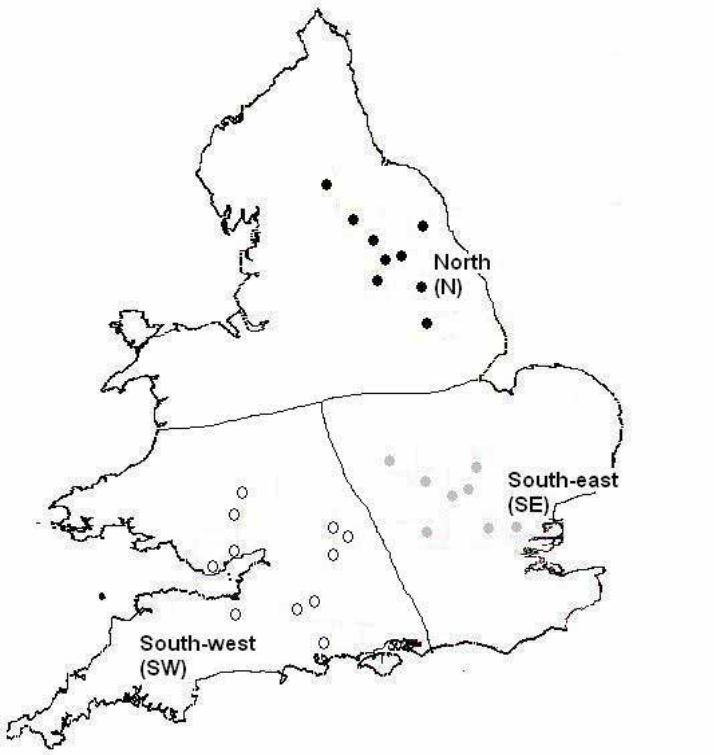

Figure 2. Location of sites from which winter oilseed rape stems were sampled in July 2003

Table 1. Geographical location of winter oilseed rape crops sampled in July 2003 and number of *L. maculans* and *L. biglobosa* isolates obtained

		Number of isolates	
Area[1]	**no. of crops sampled**	***L. maculans***	***L. biglobosa***
SE	8	30	41
SW	11	18	8
N	9	12	11
	Total	**60**	**60**

[1] see Figure 2. for location of sites and definition of areas

Effects of fungicide on populations of* L. maculans *and* L. biglobosa *on winter oilseed rape

A field experiment was done at Rothamsted in 2002/2003. Winter oilseed rape cv. Apex was sown at 80 seeds/m^2 in a randomised block design with three replicates (20 x 3m plots). Flusilazole + carbendazim was applied as Punch C at 0.8 l/ha (300g a.i./ha) on two occasions during the growing season: the 'early' treatment was sprayed on 4 November 2002 and the 'late' treatment applied on 16 January 2003. Ascospore release was monitored using a Burkard 7-day recording spore sampler (Burkard Manufacturing Co., UK), operating in open ground and surrounded by 10 Hessian-sack lined trays (45 × 75cm) placed in a circle of 10m diameter around the sampler. Each tray contained approximately 30 stems showing symptoms of colonization by *L. maculans* (stem canker) and *L. biglobosa* (upper stem lesion). The number of ascospores/m^3 air was calculated after counting the spores deposited on the Melinex tape, using a light microscope. Disease development in the crop was monitored in crop samples at

monthly intervals, when phoma leaf spotting was recorded as percentage of plants affected in untreated plots.

On 2 July 2003, approximately 2 weeks before harvest, stem canker incidence for each treatment was determined. 30 plants per treatment (10 plants per plot) were sampled and the presence or absence of phoma stem canker recorded. In addition, stem canker severity was determined. For this, 60 diseased plants per treatment (20 plants per plot) were sampled and assessed for disease symptoms. Stem canker was recorded on a 0 (no disease) to 5 (plant dead) scale, and small pieces (5 x 5mm) were sampled from the stem base cortex, stem base pith and upper stems (>10cm from stem base). These pieces were halved and one half used for fungal isolation according to method described in West et al. (2002). The other half of each sample was placed in a sterile 1.5ml Eppendorf tube and freeze-dried. DNA was extracted from the freeze-dried tissue according to the protocol described by (Fraaije et al. 1999). Polymerase Chain Reactions were done in 96-well plates (ABGene, UK) using 1µl of undiluted DNA extraction product, 200µM dNTPs, 1.5mM $MgCl_2$, 25 pmol each of the *L. maculans* and *L. biglobosa* specific forward primers LmacA and LmacB and 50 pmol of the common reverse primer LmacRev (unpublished sequences, S. Foster), 0.2 U of *Taq* DNA polymerase (RedHot Taq, ABGene, UK) and 1.5µl of 10x reaction buffer supplied with the enzyme. Sterile distilled water was added to adjust the final volume to 15µl and PCR cycling done using a GeneAmp2700 PCR System (Applied Biosystems) and a 'hot start' programme of 95°C for 2 min followed by 40 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C. Following an extension period of 10 min at 72°C, the PCR fragments were detected on 2% agarose gels by staining with ethidium bromide. Pictures were taken using the GeneGenius Imaging System (Syngene).

Results and discussion

Fungicide sensitivity of L. maculans *and* L. biglobosa *isolates from England and Wales*

In these experiments, isolates of *L. maculans* and *L. biglobosa* were more sensitive to flusilazole than tebuconazole, and significantly higher doses of fungicides were required to inhibit growth of *L. biglobosa* as compared to *L. maculans*.

When results obtained from both *L. maculans* and *L. biglobosa* isolates were analysed together, mean ED_{50} values of 0.31 and 1.06µg/ml were calculated for flusilazole and tebuconazole, respectively. When considered as two groups, the ED_{50} values differed significantly between *L. maculans* and *L. biglobosa*, for both fungicides (flusilazole: 0.27 and 0.34µg/ml, P=0.002, SED=0.023; tebuconazole: 0.67 and 1.45µg/ml, P=<0.001, SED=0.049). ED_{50} values ranged from 0.06 – 0.7µg/ml and 0.16 - 0.9µg/ml flusilazole and 0.4 - 0.94µg/ml and 0.75 – 2.63µg/ml tebuconazole for *L. maculans* and *L. biglobosa*, respectively. The ED_{50} values obtained for *L. maculans* with flusilazole fitted a double-Gaussian distribution curve, with 53% of isolates having an ED_{50} value of ≤0.2µg/ml and 23% of isolates with ED_{50} values of 0.4 – 0.6µg/ml (Fig. 3). The ED_{50} values for *L. biglobosa* with flusilazole fitted a log-Gaussian curve, with 73% of isolates having an ED_{50} value of 0.2 – 0.4µg/ml and no secondary peak. However, 3% of isolates had an ED_{50} value more than double that of most *L. biglobosa* isolates (Fig. 3).

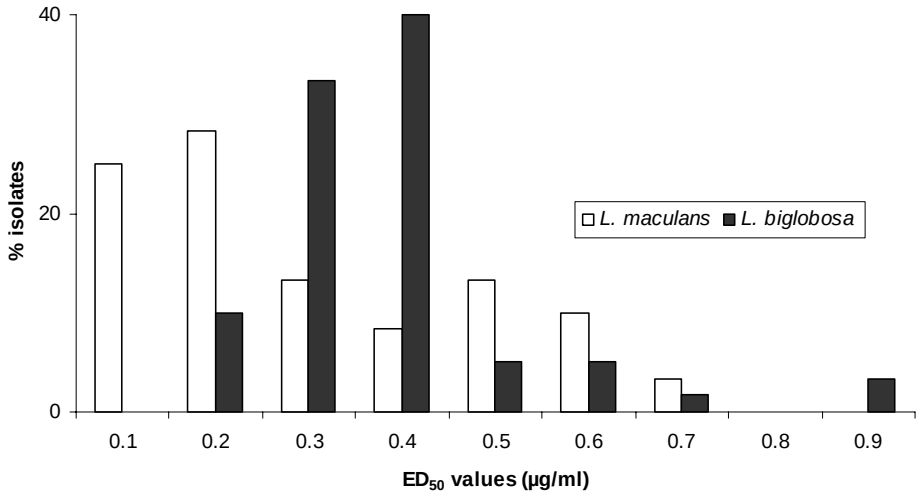

Figure 3. Distribution of ED_{50} values of *L. maculans* and *L. biglobosa* isolates tested on flusilazole

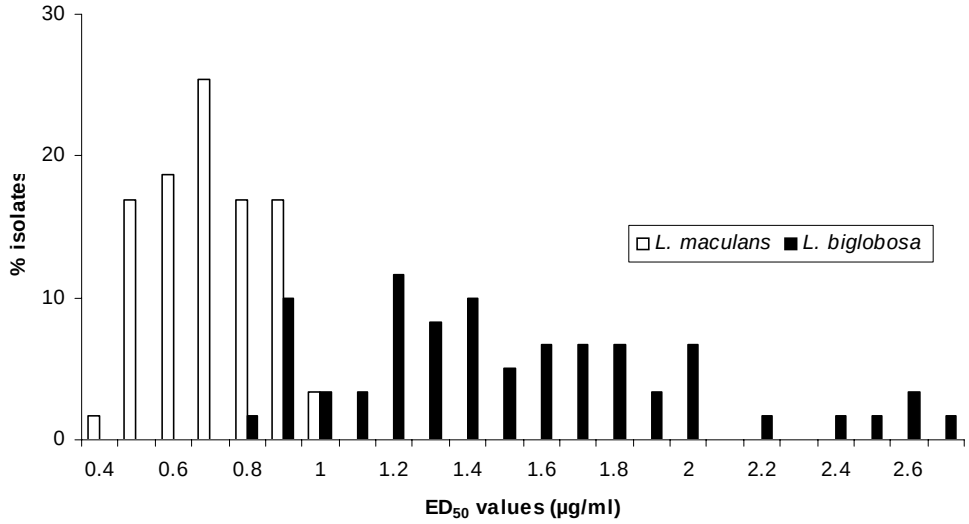

Figure 4. Distribution of ED_{50} values of *L. maculans* and *L. biglobosa* isolates tested on tebuconazole

For *L. maculans* with tebuconazole, a normal distribution of ED_{50} values was observed (Fig. 4). For 26% of isolates the ED_{50} value ranged between 0.6-0.7µg/ml and 36% and 35% of isolates had ED_{50} values of 0.4-0.6 and 0.7-0.9µg/ml, respectively. The growth response of *L. biglobosa* on tebuconazole varied (Fig. 4), only 53% of isolates had an ED_{50} value close or below the calculated mean (1.45µg/ml), and 7% of isolates had ED_{50} values more than double the mean.

A difference in sensitivity to fungicides between *L. maculans* and *L. biglobosa* has previously been noted (Cavelier et al. 1999). They used four *L. biglobosa* isolates and reported a greater mean ED_{50} value for *L. biglobosa* than for *L. maculans* when tested on flutriafol. However, the opposite was found for difenconazole, prochloraz and azoxystrobin, where an ED_{50} value for *L. biglobosa* less than that for *L. maculans* was reported.

Effects of fungicide on populations of* L. maculans *and* L. biglobosa *on winter oilseed rape

The first major *Leptosphaeria* ascospore release (>20 spores/m^3) was recorded on 21 October 2002 and ascospore discharge continued until 5 January 2003 (Fig. 5). After this time, significant ascospore discharge was recorded on five more days before the spore sampler was stopped on 19 July 2003. The early fungicide treatment was applied 2 weeks after ascospore release, when approximately 10% of plants had phoma leaf spots. The late fungicide treatment was applied in January, when 100% of plants had phoma leaf spots, after most ascospores had been discharged (Fig. 5).

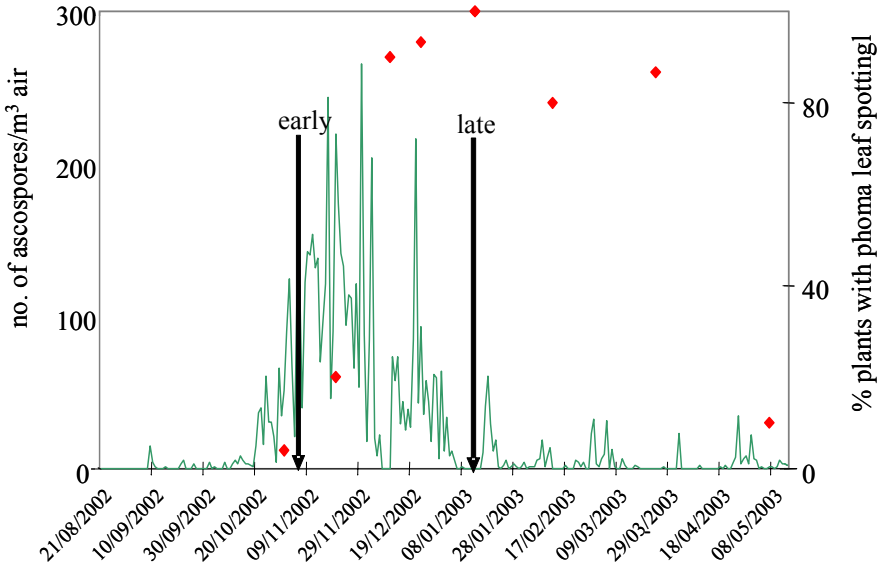

Figure 5. Timing of fungicide applications (arrows) in relation to *Leptosphaeria* ascospore release and phoma leaf spotting in winter oilseed rape at Rothamsted, 2002-2003

There was a difference in stem canker incidence between untreated and treated plots. 97% of plants were affected by stem canker if plants were untreated, whereas fungicide treatments reduced stem canker incidence to 77% of plants affected in the early treatment and 73% plants affected in the late treatment. There was also a difference in stem canker severity between untreated plots (mean canker rating of diseased plants, CR = 2.3), plots treated in November (CR = 1.9) and plots treated in January (CR = 1.6). However, no difference in yield between the different treatments was observed.

Of the two methods used for pathogen identification, PCR was more reliable, allowing positive identification of the causal pathogen for 88% of basal stem cankers and 95% of upper stem lesions. In comparison, traditional culturing methods allowed pathogen identification in 31% of basal stem cankers and 64% of upper stem lesions. Furthermore, PCR analysis identified the presence of both *L. maculans* and *L. biglobosa* in 7% of the basal stem cankers sampled, whereas in culture, only one of the two pathogens was observed. In this experiment, the timing of fungicide application had an effect on the pathogen populations. The November fungicide treatment significantly reduced the number of *L. biglobosa* isolations made from both basal stem canker and upper stem lesions (Fig. 6).

The frequencies of *L. maculans* and *L. biglobosa* isolates from different stem tissues were similar to those in previous reports of differential tissue colonization by the two pathogens (West et al. 2002), although the number of *L. biglobosa* isolates obtained from the stem pith (33% and 27% from control and late treated samples) was greater than the 9% reported by West et al. (2002).

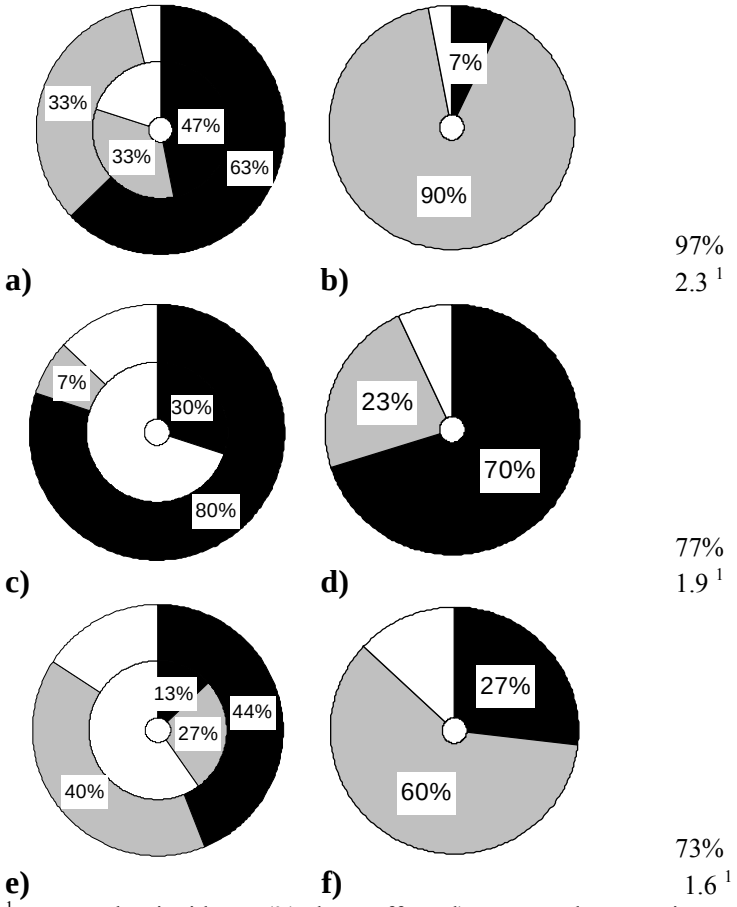

Figure 6. Proportions of *L. maculans* (black areas), *L. biglobosa* (grey areas) and 'other' fungal (white areas) isolates obtained from oilseed rape stems treated with no fungicide (control) (**a, b**), early fungicide application (**c, d**) and late fungicide application (**e, f**). Samples were taken from basal stem cankers (**a, c, e**) (outer circle – cortex; inner circle – pith) or upper stem lesions (**b, d, f**) (>10cm from stem base) just before harvest

In these experiments we aimed to investigate possible differential effects fungicides have on *L. maculans – L. biglobosa* populations. The *in vitro* experiment showed a difference in sensitivity to triazole fungicides of the two pathogens. The field experiment suggested that fungicide application can have an effect on pathogen distribution within the plant. Further work is currently being done, to confirm these results and to investigate more details of differences in response to fungicides between *L. maculans* and *L. biglobosa*.

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Molecular characterization of Portuguese isolates of *Leptosphaeria maculans* using PCR-ISSR and RAPD markers

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Abstract: PCR-ISSR and RAPD markers were used to study the genetic relationships of 30 isolates of *Leptospheria maculans* including 18 Portuguese isolates from Beja region (south Portugal). Foreign isolates were also included to determine the relationships of Portuguese isolates to other related isolates. Cluster and principal components analyses were conducted using a package of computer programs and PCR-ISSR and RAPD data from amplification with selected ISSR primers and RAPD markers, detecting 234 polymorphic fragments. The results showed that the 30 isolates clustered into two distinct groups (Tox^+ and Tox^0 isolates) and 4 subgroups: i) a large and compact subgroup containing all the Tox^+ *L. maculans* "brassicaceae" isolates including all the Portuguese isolates; ii) the unique "Lepidium" Tox^+ isolate; iii) a relatively heterogeneous subgroup with the Tox° NA2 *L. biglobosa* "canadensis" isolates; and iv) a dispersed subgroup with the other Tox° NA1 and NA3 *L. bigolosa* isolates and NA2 *L. bigolosa* "erysimii" isolate. There is low similarity between these three isolates. The Portuguese and foreign Tox^+ *L. maculans* "brassicaceae" isolates could be further divided into phenetic groupings/clusters. These groupings of Portuguese isolates based on PCR-ISSR and RAPD data do not corresponded to their pathogenicity groups revelead by plant differentials.

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Thlaspi arvense, a source of A-type isolates of *Phoma lingam*?

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Abstract: Blackleg or stem canker, caused by the fungus *Phoma lingam*, is a serious disease of oilseed rape (*Brassica napus*) around the world. Numerous strains of the fungus have been isolated from oilseed rape (OSR) and characterized. In contrast, only limited information is available regarding isolates from cruciferous weeds like stinkweed (*Thlaspi arvense*), hedge mustard (*Sisymbrium officinale*), *Lepidium* spp., *Erysimum* spp. and others that occur in oilseed producing regions. Most isolates described so far resemble B-type isolates classified to be non-aggressive on *B. napus*; B-type isolates do not produce host nonspecific toxins called sirodesmins (SIRO°, Tox°), which belong to the chemical family of dioxo-piperazines. In contrast, A-type isolates are classified to be aggressive on oilseed rape, producing sirodesmins ($SIRO^+$, TOX^+). A-type isolates are associated with stem canker and are believed to have a major impact on yield of OSR compared to B-type isolates. Previous studies have suggested that cruciferous weeds may serve as a green bridge for A-type isolates and may, therefore, be important with respect to epidemiology of *P. lingam*. The objective of this study was to investigate 18 isolates obtained from seeds of Canadian accessions of *T. arvense*. These 'Thlaspi' isolates were characterized by studying phenotypic and genotypic traits and compared with nine reference isolates from the *International Blackleg of Crucifers Network* (IBCN) collection displaying the known variability of *P. lingam*.

Radial growth of mycelium was investigated on both V8-juice agar (V8) and malt extract agar (MEA). Growth rates of the 'Thlaspi' isolates displayed a continuum (mean values on V8/MEA: 0.71/0.45 cm/d) between both A-type (0.53/0.23 cm/d) and B-type NA1 (0.75/0.46 cm/d) reference isolates. Some 'Thlaspi' isolates also grew slightly faster than a B-type NA1 isolate on MEA. All 'Thlaspi' isolates produced various colored pigments in Czapek-Dox broth. For the 'Thlaspi' isolates, no *in vitro* production of sirodesmins was detected using thin layer chromatography. On the basis of these phenotypic characters, 'Thlaspi' isolates are related to B-group isolates. VNTR-PCR, ERIC-PCR and ITS analyses revealed two 'Thlaspi' groups, which are distinct from IBCN A-group, B-group (NA1, NA2, NA3, 'Thlaspi', 'Sisymbrium', 'Erysimum', Australia) and 'Lepidium' reference isolates. Finally, pathogenicity tests on OSR cv. Lirabon (blackleg susceptible) were performed. None of the 'Thlaspi' isolates was virulent on cotyledons of Lirabon. Aggressiveness of 'Thlaspi' and IBCN reference isolates also was compared on stems of Lirabon. All 'Thlaspi' isolates were significantly less aggressive than the A-type reference isolate. In conclusion, there is no evidence from this study that *T. arvense* serves as a reservoir for A-type isolates, although evaluation of more *P. lingam* isolates from *T. arvense* growing in canola fields would be desirable.

Material and methods

Eighteen isolates of *Phoma lingam* were recovered from seeds of the cruciferous weed *Thlaspi arvense* collected from various locations in Saskatchewan, Canada. The 'Thlaspi' isolates were studied together with nine well characterized isolates from the *International Blackleg of Crucifers Network* (IBCN) collection representing the known genotypic diversity of *P. lingam* (Table 1). Of the IBCN reference isolates, one belonged to the A-type group and seven to various B-type groups. IBCN 84 is an exceptional isolate originating from a cress

weed and which produces both pigments and sirodesmins; it is genetically distinct from A-type and B-type isolates.

Mycelial growth rates of the isolates were compared on V8-juice agar (V8) and malt extract agar (MEA). Three replicate plates per isolate were incubated at 20°C in the dark. Colony growth was measured twice at a 90° angle. The average colony diameter for each isolate was recorded daily.

Pigment and sirodesmin production was investigated on cultures grown in Czapek-Dox broth amended with 2% yeast extract and 2% peptone. Water soluble pigments were examined as described by MCGEE & PETRIE (1978). Sirodesmin production was studied by thin layer chromatography as described by KOCH *et al.* (1989).

Pycnidiospores were produced on Czapek-Dox agar. Suspensions of $1x10^7$ spores/ml were prepared by dilution in sterile distilled water. Suspensions were stored in 1.5 ml Eppendorf tubes at –20°C until required.

Table 1. Origin and properties of the isolates used in this study.

(A) 'Thlaspi' isolates

Isolate #	Map location	Collected from, collected in	Year
Thl-10–13	*AAFC-Melfort Research Farm*	*stinkweed seeds, canola plots*	*2000*
Thl-14–23	*Site 001, Eastend, Saskatchewan*	*stinkweed seeds, barley field*	*2000*
Thl-24–27	*Site 003, Eastend, Saskatchewan*	*stinkweed seeds, road allowance*	*2000*
Thl-28	*Site 004, Eastend, Saskatchewan*	*stinkweed seeds, barley field*	*2000*

(B) IBCN reference isolates

Isolate #	Properties*	Collected from, country**, originator	Year
IBCN 02	*A; SIRO$^+$; PIG°; aggressive*	*B. napus, D, HH Hoppe*	*1992*
IBCN 26	*NA1; SIRO°; PIG$^+$; non-aggressive*	*B. napus, PL, Karolewski*	*1991*
IBCN 30	*NA-Aus; SIRO°; PIG$^+$; non-aggressive*	*B. napus, AUS, B Howlett*	*1991*
IBCN 64	*Thlaspi; SIRO°; PIG$^+$; non-aggressive*	*T. arvense, CAN, G Séguin-Swartz*	*1993*
IBCN 82	*Sisymbrium; SIRO°; PIG$^+$; non-aggressive*	*B. juncea, CAN, G Séguin-Swartz*	*1992*
IBCN 83	*Erysimum; SIRO°; PIG$^+$; non-aggressive*	*Erysimum sp., CAN, G Séguin-Swartz*	*1995*
IBCN 84	*Lepidium; SIRO$^+$; PIG$^+$*	*Lepidium sp., CAN, G Séguin-Swartz*	*1992*
IBCN 91	*NA3; SIRO°; PIG$^+$; non-aggressive*	*B. oleracea, USA, PH Williams*	
IBCN 92	*NA2; SIRO°; PIG$^+$; non-aggressive*	*B. rapa, CAN, PH Williams*	

* Genotype; sirodesmin production; pigment production; virulence on winter oilseed rape.
** D = Germany, PL = Poland, AUS = Australia, CAN = Canada, USA = United States.

Aggressiveness of the 'Thlaspi' isolates was investigated on *Brassica napus* cultivar Lirabon (Deutsche Saatveredelung, Lippstadt, Germany), which is susceptible to *P. lingam* (KUSWINANTI *et al.*, 1999). Isolates IBCN 02 (A-type) and IBCN 26 (B-type NA1) were chosen as the aggressive and non-aggressive reference isolates for this study. Pathogenicity tests were conducted in a controlled environment chamber. Cotyledons were punctured with a needle and inoculated with a 10 µl drop of spore suspension. Symptoms were scored 14 dpi using the IMASCORE rating scale (VOLKE, 2000).

Stems were inoculated in a greenhouse experiment at growth stage BBCH 33–35. Stems were punctured above the petiole of the first true leaf and a mycelium plug was attached to the wound. Inoculated plants were transferred to a foliar tunnel for 72 h. Plants were then incubated in a greenhouse at 23°C and a 16 h photoperiod. Disease severity was scored 49 dpi

using a slightly modified rating scale described by KUTCHER *et al.* (1993). Lesion length, girdling and depth were used to calculate the volume of diseased tissue (VDT) from 17 individual plants and disease severity was classified using 0–9 scales as follows: ***Lesion Length:*** 0 = no infection; 1 = 1–4 mm; 2 = 5–8 mm; 3 = 9–12 mm; 4 = 13–16 mm; 5 = 17–20 mm; 6 = 21–24 mm; 7 = 25–28 mm; 8 = 29–32 mm; 9 = >32 mm; ***Girdling and Depth:*** 0 = no infection; 1 = >0–11%; 2 = 12–22%; 3 = 23–33%; 4 = 34–44%; 5 = 45–55%; 6 = 56–66%; 7 = 67–77%; 8 = 78–88%; 9 = >88%; HR = 1–D/9; VDT = $(1–HR^2)$ * G/9 * L.

DNA analysis: Total DNA was prepared from either freshly grown or freeze dried mycelium. DNA was extracted using the DNeasy Plant Mini Extraction Kit (QIAGEN, Hilden, Germany). DNA analysis was performed according to standard procedures (SAMBROOK & RUSSELL, 2000). Isolates were characterized by VNTR- and ERIC-fingerprinting. For both techniques, about 25 ng of template DNA was used in a final volume of 25 µl comprising 0.2 mM dNTPs, 1 µM of each primer, 1.25 U Taq polymerase (TaKaRa Bio Europe, Gennevilliers, France), 1.5 mM $MgCl_2$ and 1X concentrated buffer supplied by the polymerase manufacturer. The cycler T-Gradient (Biometra, Göttingen, Germany) was used for these studies. For VNTR-PCR, the primer 5´-*ggt.ggc.ggc.tct*-3´ was used (HEATH *et al.*, 1993). The PCR profile was: 30 cycles, denaturation for 2 min at 94°C, annealing for 1 min at 57°C and extension for 3 min at 72°C. For ERIC-PCR, the primers Eric1R (5´-*atg.taa.gct.cct.ggg.gat.tac.c*-3´) and Eric2 (5´-*aag.taa.gtg.act.ggg.gtg.agc.g*-3´) were used as described by GILLINGS & HOLLEY (1997) and WEINGART & VÖLKSCH (1997). The profile of ERIC-PCR was: 36 cycles, denaturation for 2 min at 94°C, annealing for 1 min at 52°C and extension for 3 min at 72°C. Internal Transcribed Spacer (ITS) regions were amplified using the primers ITS4 (5´-*tcc.tcc.gct.tat.tga.tat.gc*-3´) and ITS5 (5´-*gga.agt.aaa.agt.cgt.aac.aag.g*-3´) described by WHITE *et al.* (1990). The PCR was conducted using the following profile: denaturation for 1 min at 94°C, annealing for 1 min at 54°C and extension for 1 min at 72°C. PCR products were either separated on standard agarose gels or by PAGE gel electrophoresis.

Data analysis: Genetic similarities were calculated using the similarity coefficient of DICE (1945). Data were clustered using the *Unweighted Pair Group Method with Arithmetic Mean* (UPGMA) algorithm. Both Gel ComparII (Applied Maths, Sint-Martens-Latem, Belgium) and SYN-TAX 5.1 (PODANI, 1993) were used to construct the dendrograms.

Results and discussion

Oilseed rape is mainly grown in 3–4 year rotations in Europe and Canada. Decomposition of stubble bearing pseudothecia and pycnidia of the stem canker pathogen is reported to take two years in humid areas (WEST *et al.*, 2001). ALABOUVETTE & BRUNIN (1970) reported the production of ascospores from oilseed rape residues buried for four years, but stubble from the last rotation probably contributes to disease pressure only when conditions are unfavorable for decomposition. The primary inoculum is believed to be represented by airborne ascospores released from pseudothecia formed on oilseed rape residues in neighbouring fields. These ascospores are carried by wind for long distances (WILLIAMS, 1992). Other sources of inoculum also may be responsible for new epidemics. Infected seed may play a role (GABRIELSON, 1983), and cruciferous weeds may serve as endemic sources of the pathogen (PETRIE *et al.*, 1995). It is well known that *P. lingam* can be isolated from *Sisymbrium* spp., *Erysimum* spp., *T. arvense* and other crucifers (PETRIE, 1969; GUGEL & PETRIE, 1992; PETRIE *et al.*, 1995; ROUXEL *et al.*, 1995). Typical pale, necrotic lesions surrounded by a dark border and resembling lesions on oilseed rape can be seen on *T. arvense* in the field. Numerous pycnidia may be present at the centers of these lesions (Figure 1).

Figure 1. Phoma lesions formed on lower (left) and upper (right) stems of *Thlaspi arvense*.

Table 2. Growth rates of *Phoma lingam* isolates cultured at 20°C in the dark on V8-juice agar (V8) and malt extract agar (MEA).

Group	Isolate #	Growth rates on V8		MEA	
		(cm/d)	mean (cm/d)	(cm/d)	mean (cm/d)
A-type	*IBCN 02*	*0.53*	*0.53*	*0.23*	*0.23*
B-type NA1	*IBCN 26*	*0.75*	*0.75*	*0.46*	*0.46*
Thlaspi	*Thl-10*	*0.76*		*0.45*	
Thlaspi	*Thl-11*	*0.75*		*0.42*	
Thlaspi	*Thl-12*	*0.71*		*0.46*	
Thlaspi	*Thl-13*	*0.72*		*0.54*	
Thlaspi	*Thl-14*	*0.73*		*0.55*	
Thlaspi	*Thl-15*	*0.72*		*0.53*	
Thlaspi	*Thl-16*	*0.70*		*0.52*	
Thlaspi	*Thl-17*	*0.75*		*0.48*	
Thlaspi	*Thl-18*	***0.60***	*0.71*	***0.24***	*0.45*
Thlaspi	*Thl-19*	***0.65***		***0.25***	
Thlaspi	*Thl-20*	*0.74*		*0.52*	
Thlaspi	*Thl-21*	*0.72*		*0.56*	
Thlaspi	*Thl-22*	*0.73*		*0.55*	
Thlaspi	*Thl-23*	*0.75*		*0.52*	
Thlaspi	*Thl-24*	*0.75*		*0.30*	
Thlaspi	*Thl-26*	*0.70*		*0.40*	
Thlaspi	*Thl-27*	*0.73*		*0.56*	
Thlaspi	*Thl-28*	***0.60***		***0.32***	

The objective of our study was to characterize a small collection of *P. lingam* isolates recovered from seeds of different accessions of *T. arvense* (Table 1). Earlier studies of A- and B-type isolates of *P. lingam* revealed that these isolates differ in numerous traits (WILLIAMS & FITT, 1999; HOWLETT *et al.*, 2001). Growth rates on specific media are a potentially discriminative trait (CUNNINGHAM, 1927; POUND, 1947). These differences become more pronounced if isolates are grown on nutrient poor media. We measured radial growth rates of the 'Thlaspi' isolates and compared them with two reference isolates from the IBCN collection. These reference isolates represented the A-type and B-type NA1 strains. Growth rates of all isolates were higher on V8 than on MEA (Table 2), but differences among isolates were more pronounced on MEA. A-type isolate IBCN 02 displayed the weakest growth, which amounted to half the growth rate of B-type isolate IBCN 26. Most of the 'Thlaspi' isolates behaved similarly to IBCN 26. There were only three 'Thlaspi' isolates (Thl-18, 19, 28; values in bold) that grew significantly more slowly, both on V8 and MEA. Isolate Thl-24

(values underlined) showed reduced growth on MEA, but growth on V8 was similar to that of the other 'Thlaspi' isolates.

HUANG *et al.* (2001) showed that ascospore growth patterns differed among A- and B-type isolates. Differences observed using the parameter radial growth on solid media may be explained by the more branched mycelial growth of A-type isolates. However, whether radial growth is a good parameter for biomass production may be answered by experiments comparing growth rates in liquid media measuring mycelial dry matter.

Isolates were tested for virulence on *B. napus*. Pycnidiospore suspensions were used for inoculating punctured cotyledons of the susceptible cultivar Lirabon, which is part of a differential set described by KUSWINANTI *et al.* (1999). Most of the isolates were non-aggressive and the host plants generally responded with hypersensitive-like reactions. Only in a few cases were intermediate reactions observed, and these were characterized by dark, slightly larger lesions that were not characteristic of a susceptible interaction, i.e., pronounced greyish-green collapsed tissue bearing numerous pycnidia, as noted for IBCN 02 (Table 3).

Table 3. Pigmentation, sirodesmin production and virulence of *Phoma lingam* 'Thlaspi' isolates and IBCN A-type and B-type NA1 reference isolates.

Group	Isolate #	Pigment color	Sirodesmin production	Symptoms on cotyledons of *Brassica napus* cv. Lirabon
A-type	*IBCN 02*	*No pigment*	*SIRO*$^{+}$	*Grey-green tissue collapse, abundant sporulation*
B-type NA1	*IBCN 26*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-10*	*Light brown*	*SIRO°*	*Intermediate*
Thlaspi	*Thl-11*	*Brown*	*SIRO°*	*Intermediate*
Thlaspi	*Thl-12*	*Brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-13*	*Yellowish*	*SIRO°*	*HR-like*
Thlaspi	*Thl-14*	*Yellowish*	*SIRO°*	*HR-like*
Thlaspi	*Thl-15*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-16*	*Brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-17*	*Yellowish*	*SIRO°*	*HR-like*
Thlaspi	*Thl-18*	*Light brown*	*SIRO°*	*Intermediate*
Thlaspi	*Thl-19*	*Brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-20*	*Light brown*	*SIRO°*	*Intermediate*
Thlaspi	*Thl-21*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-22*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-23*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-24*	*Red brown*	*SIRO°*	*Intermediate*
Thlaspi	*Thl-26*	*Brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-27*	*Light brown*	*SIRO°*	*HR-like*
Thlaspi	*Thl-28*	*Light brown*	*SIRO°*	*HR-like*

Differential sets of *B. napus* have been developed by several research groups (BADAWY *et al.*, 1991; MENGISTU *et al.*, 1991; KUSWINANTI *et al.*, 1999). Cotyledon assays rely more or less on monogenic resistance, as shown by segregation analysis (ANSAN-MELAYAH *et al.*, 1998 and others). Adult plant resistance is believed to be an oligogenic or polygenic trait and not necessarily related to resistance of plants at juvenile stages. It may also, therefore, be important to study the aggressiveness of isolates on stem tissue. All chosen IBCN reference and 'Thlaspi' isolates were inoculated on stems using a mycelium plug technique. A significant difference between A-type IBCN 02 and all other tested isolates was observed (Figure 2). IBCN 02 produced deep lesions on stems, whereas the other isolates caused only small, superficial lesions.

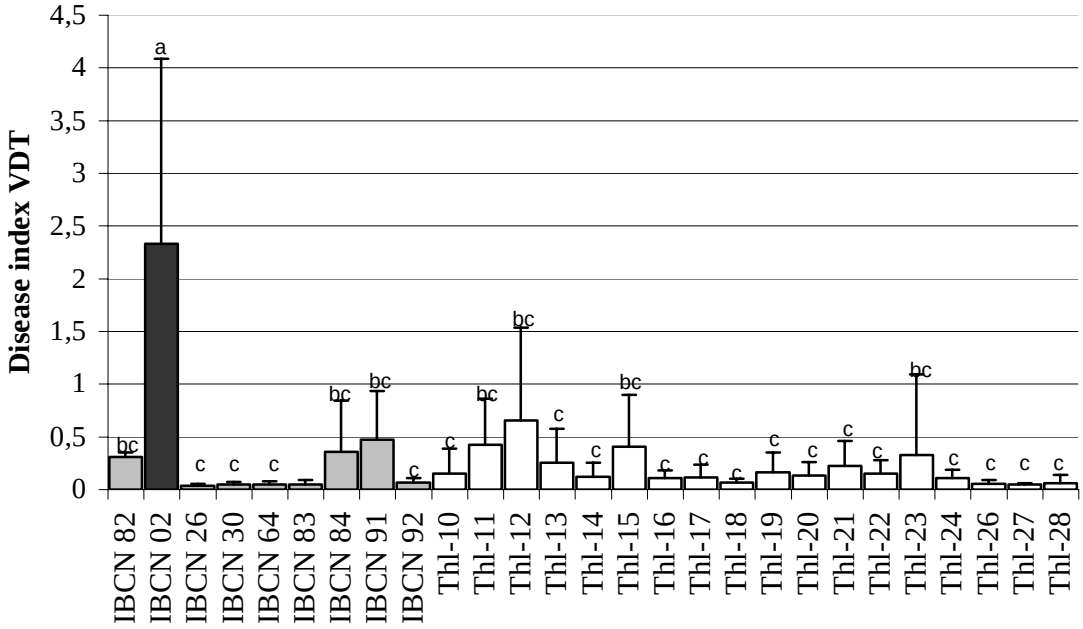

Figure 2. Volume of diseased tissue (VDT, KUTCHER *et al.*, 1993) on stems of *Brassica napus* cv. Lirabon 49 days post-inoculation with IBCN reference isolates (IBCN 02, 26, 30, 64, 82, 83, 84, 91, 91) and 'Thlaspi' isolates (Thl-10–24, Thl-26–28). Different letters indicate statistically significant differences (F test, P <0.05).

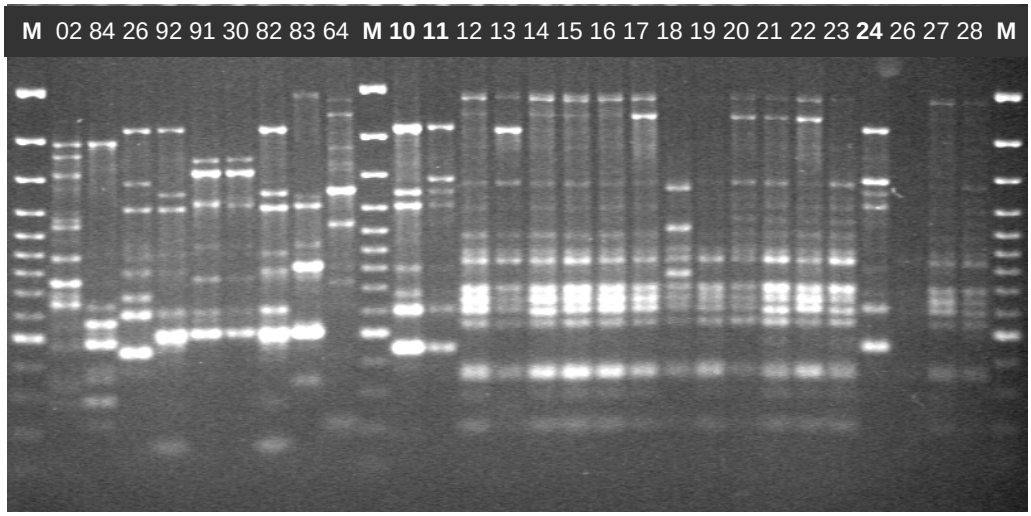

Figure 3. VNTR-PCR fingerprint analysis of *Phoma lingam* isolates. M: 100 bp DNA ladder plus (3, 2, 1.5, 1.2, 1.031, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kbp; MBI, Fermentas, St. Leon Roth, Germany). Lane 1: M, lanes 2–10: IBCN isolates (*as indicated*), lane 11: M, lanes 12–29: 'Thlaspi' isolates (*as indicated*), lane 30: M.

Isolates IBCN 82 from *Erysimum* sp., IBCN 84 from *Lepidium* sp., NA2 reference isolate IBCN 91 and 'Thlaspi' isolates Thl-11, 12, 15 and 23 caused slightly higher disease severity (>0.25 VDT) than other B-type isolates, although the differences were not statistically significant (Figure 2). Of these isolates, only Thl-11 also caused an intermediate reaction on cotyledons (Table 3). All other 'Thlaspi' isolates causing intermediate reactions on cotyledons

(Thl-10, 18, 20, 24) were clearly non-aggressive on stems. Thus, the ‘Thlaspi’ isolates evaluated in this study can be classified as non-aggressive on *B. napus*.

Additional experiments were performed to elucidate the relatedness of the ‘Thlaspi’ isolates to known B-type isolates. For this purpose three molecular approaches were chosen to investigate similarities to a set of IBCN reference isolates which displayed the known genetic variability of *P. lingam* (KOCH *et al.*, 1991; BALESDENT *et al.*, 1998; PURWANTARA *et al.*, 2000; KOOPMANN *et al.*, 2003; MENDES-PEREIRA *et al.*, 2003). VNTR- and ERIC-PCR fingerprints have previously been used to study diversity within the IBCN collection (KOOPMANN *et al.*, 2003). In this study, VNTR-PCR displayed amplicon patterns comprising 6–13 polymorphic bands; amplicon size ranged from 200–3000 bp (Figure 3). None of the ‘Thlaspi’ isolates (aligned between the central and right marker lanes) showed similarities to the IBCN reference isolates (aligned between the left and central marker lanes), nor to IBCN 64 (lane 10), which also was isolated from *T. arvense*. Furthermore, two typical patterns were observed among the ‘Thlaspi’ isolates: Thl-10, 11 and 24 formed a unique group (Group I), whereas the other isolates belonged to a separate group (Group II). These groupings were also achieved using ERIC-PCR (Figure 4). ‘Thlaspi’ Group I isolates clustered together and were most similar to IBCN 26 (B-type NA1) and IBCN 30 (B-type, NA-Aus). ‘Thlaspi’ Group II, comprising most of the ‘Thlaspi’ isolates examined, appeared as an outgroup and displayed more similarity to some of the other IBCN isolates than to the ‘Thlaspi’ Group I isolates. IBCN 64 also showed only poor similarity to the other ‘Thlaspi’ isolates.

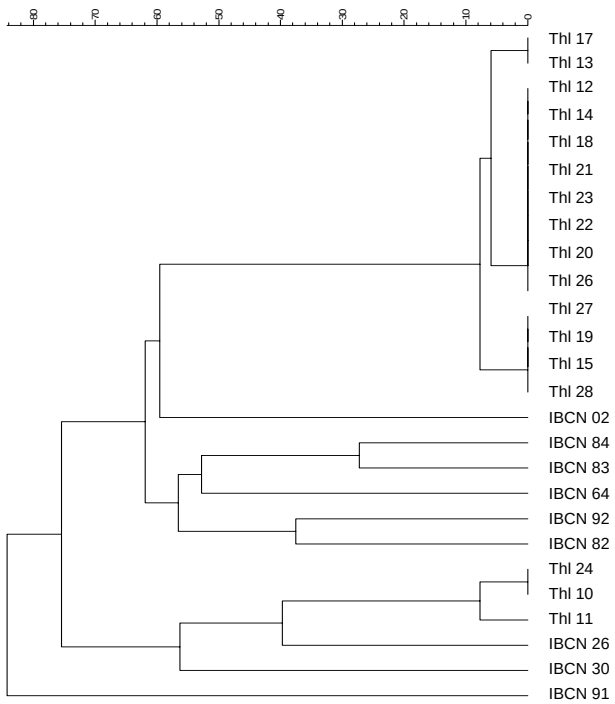

Figure 4. Dendrogram generated via UPGMA based on dissimilarity (DICE, 1943) data of an ERIC- PCR fingerprint analysis of *Phoma lingam* IBCN reference isolates and ‘Thlaspi’ isolates.

Examination of ITS amplicon sizes also supported the results of the PCR fingerprint studies. Figure 5 shows IBCN 64 and representative ‘Thlaspi’ isolates between the central and right marker lanes. Amplicons of isolates in ‘Thlaspi’ Group I (Thl-10, 11, 24) differed slightly in size compared to those of isolates in ‘Thlaspi’ Group II (Thl-13, 14, 15); IBCN 64 also was significantly different from both ‘Thlaspi’ Group I and Group II isolates.

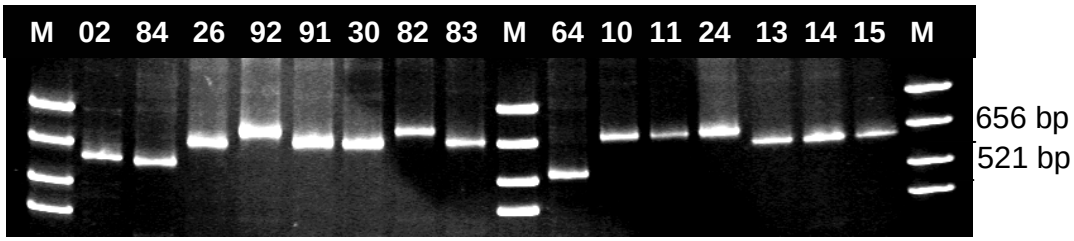

Figure 5. Length polymorphisms of ITS-rDNAs of *Phoma lingam* isolates separated on a 5–10% polyacrylamide gradient gel (350 V, 3.5 h). M: marker pBR322/*Alu*I, left: IBCN reference isolates (IBCN 02, 84, 26, 92, 91, 30, 82, 83), right: IBCN 64 and 3 representative isolates of 'Thlaspi' fingerprint groups I (Thl-10, 11, 24) and II (Thl-13, 14, 15).

In conclusion, the 'Thlaspi' isolates evaluated in this study resemble B-type isolates on the basis of pigment production, sirodesmin production, mycelial growth rate and virulence on *B. napus* seedlings and adult plants. Results of the virulence tests suggest that isolates of *P. lingam* from *T. arvense* are not likely to enhance the risk of stem canker epidemics in canola fields. Although isolates aggressive to canola have been recovered from *T. arvense* in western Canada (PETRIE *et al.*, 1995), none of the isolates evaluated in our study were found to be aggressive to canola. However, the number of isolates used in our study was relatively small, and it would be desirable to evaluate more *P. lingam* isolates from *T. arvense* growing in canola fields.

Genotypic traits further demonstrated that isolates of the 'Thlaspi' strain were distinct from isolates of the other *P. lingam* strains known to date. High genetic variability among 'Thlaspi' isolates is evident from the three 'Thlaspi' genotype clusters described (Group I, Group II, IBCN 64). The 'Thlaspi' isolates may belong to a different taxon, as recently shown for B-type NA1 isolates (*Leptosphaeria biglobosa*) by SHOEMAKER & BRUN (2001). Preliminary mating studies with A-type, B-type NA1 and B-type NA2 mating reference isolates have not been successful (Koopmann, unpublished). Furthermore, intraspecific crosses among 'Thlaspi' Group I and 'Thlaspi' Group II isolates have not yet been achieved. The information generated by such crosses may result in a further taxonomic revision of this species complex.

Acknowledgements

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Sirodesmins in tissues of infected rape plants revisited

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Abstract: Sirodesmin PL and other structurally related compounds are well known phytotoxins produced *in vitro* by fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not (vegetative stage: *Phoma lingam* (Tode ex Fr.) Desm.). These phytotoxins are not host-specific and their application on leaves of plants from various species causes formation of necrotic spots. However, there still is some doubt regarding the role of these compounds during the process of infection of *Brassica napus* plants. Sock et al. (1995) have found sirodesmin PL to be present in leaves of *B. napus* infected with *L. maculans*, but in these experiments plants after the inoculation had to be placed in darkness for 4 days. On the other hand, according to the studies of MSC Pedras and her team (Pedras and Biesenthal, 1998), sirodesmin PL is regarded to be not present in tissues of the infected rape plants.

This study has been performed using the HPLC instrument connected to mass spectrometer (Esquire 3000, Bruker, Germany). The high sensitivity of MS enables the detection and identification of substances present in amounts as low as 1×10^{-12} g. The plant material used were cotyledons of oilseed rape seedlings used in the Williams's test from cultivars differing in their resistance to *L. maculans* (Westar, Columbus, Darmor MX) and leaves of naturally infected plants as well as the non-infected controls. Secondary metabolites were extracted from the freeze-dried plant tissue with methanol-water solution 70% and purified using solid phase extraction columns C_{18}. The HPLC chromatography was performed in the reversed phase system using water-acetonitrile gradient elution and compounds were identified according to their ESI MS and MS/MS (up to MS^4) spectra.

The experiments demonstrated the presence of sirodesmins PL and J in cotyledons exhibiting the susceptible reaction (Westar, Columbus) but was neither found in those resistant to the infection (Darmor MX) nor in the not infected seedlings. Similarly, rape leaves with symptoms of *L. maculans* infection contained both sirodesmins which were not found in the healthy leaves.

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Agrobacterium tumefaciens-mediated transformation of *Leptosphaeria maculans*

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Abstract: *Leptosphaeria maculans* (anamorph: *Phoma lingam)*, a severe phytopathogen of oilseed *Brassica* spp causing stem canker, was transformed successfully using *Agrobacterium*-mediated transformation. The selection marker employed was the *hph* gene conferring resistance to hygromycin B. Transformation with the green fluorescent protein (GFP) gene was also performed. Transformation frequencies of up to 120 transformants per 10^7 conidia were obtained. The presence of the *hph* gene was checked by PCR and Southern analysis. The majority of *L. maculans* transformants contained a single-copy integration of the marker gene at different chromosomal sites. Transformants were mitotically and meiotically stable. GFP expression was observed in-vivo with the GFP-tagged strain. *Agrobacterium*-mediated transformation is now routinely used to generate a large collection of mutants that will allow us to identify genes that are paramount for *Leptosphaeria maculans* pathogenicity to oilseed rape.

Key words: *Leptosphaeria maculans*, *Agrobacterium tumefaciens*, Hygromycin B, Green Fluorescent Protein, Random mutagenesis

Introduction

Leptosphaeria maculans (anamorph: *Phoma lingam*) is one of the most important fungal pathogen of oilseed rape (*Brassica napus* and *B. rapa*). The infection pathway of the fungus begins with leaf infection by airborne ascospores from the seedling to the adult stage. Following the germination of ascospores, hyphae penetrate the plant tissues via stomata, without differentiating specialized penetration organs such as appressoria. The fungus then colonizes intercellular spaces between mesophyll cells and follows vascular strands to reach the petiole. This biotrophic phase is symptomless and lasts for several months, mainly in the case of winter oilseed rape, for which the endophytic stage can last up to nine months. The fungus finally invades and kills cells of the stem cortex resulting in a basal stem canker (blackleg), which can cause severe losses through lodging of the crop.

Despite its worldwide economic importance, and that of related phytopathogenic Dothideomycetes, too little is known about the molecular bases of pathogenicity and species specificity of *L. maculans*. For example, only one pathogenicity gene is known to date, cyanide hydratase (Idnurm and Howlett 2002). A better knowledge of pathogenicity determinants now necessitates both the development of efficient transformation protocols for colonisation tracking in plant or homologous recombination of candidate genes, and high throughput transformation platforms to generate large collections of mutants for phenotype alteration identification.

Historically, transformants of *L. maculans* have been obtained 16 years ago by transforming protoplasts with circular vectors in the presence of polyethylene glycol (PEG) (Farman and Oliver 1988; 1992). These studies demonstrated that the integration and

expression in the fungal genome of a selectable marker conferring resistance to hygromycin B and of two reporter genes, namely β-glucuronidase and green fluorescent protein, is possible. Later on, an improved method, the restriction enzyme mediated integration (REMI), was used to get collections of fungal transformants, including in *L. maculans* (Lu et al. 1994; Sexton and Howlett, 2001). REMI is described as an interesting mutagenesis procedure because the transforming DNA tags the mutated gene and can be subsequently used to clone the corresponding wild-type gene by plasmid rescue or polymerase chain reaction (PCR). Nevertheless, several studies performed on different fungal species have indicated that protocols necessitating the use of fungal protoplasts, including REMI have several severe drawbacks (reviewed in Mullins and Kang 2001). One is that the vector used is generally inserted at several sites in the fungal genome, therefore complicating the recovery of the single gene responsible for the altered phenotype. Moreover, the number of generated mutants that appear to be untagged by the transforming DNA can reach 20% to 50% of the mutants, since the protoplasting phase itself is able to generate large-genome recombination or deletions. Finally, the current unavailability of protoplasting enzymes (Novozyme) necessitates that new enzymatic cocktails are designed to get an efficient protoplasting phase in fungi. This is an extremely tedious and time-consuming task as we experienced it in the laboratory, with a very insufficient efficiency (J. Schmit, unpublished data). Thus, the development of new reliable and user-friendly transformation techniques is needed. As this technique has been used successfully to generate large mutant collections in plants, an alternative transformation procedure based on *Agrobacterium tumefaciens* tumor-inducing DNA (T-DNA) transfer was developed in fungi (de Groot et al. 1998), and became rapidly extremely popular due to its efficiency in numerous fungal species. Therefore, the purpose of this study was to set up agro-transformation in *L. maculans*, and evaluate the reliability, ease of use and efficiency of this transformation method, particularly to generate large mutant collections.

Materials and methods

Fungal material and culture conditions

Isolate v23.1.3 (Mat-) and v23.1.2 (Mat+) were obtained following in vitro crosses (cross # 23) (Balesdent et al. 2001). Growth and conidia production in vitro on 20% V8-juice agar were as described by Ansan-Melayah et al. (1995). In vitro crosses and random ascospore progeny recovery were performed as previously established (Gall et al. 1994).

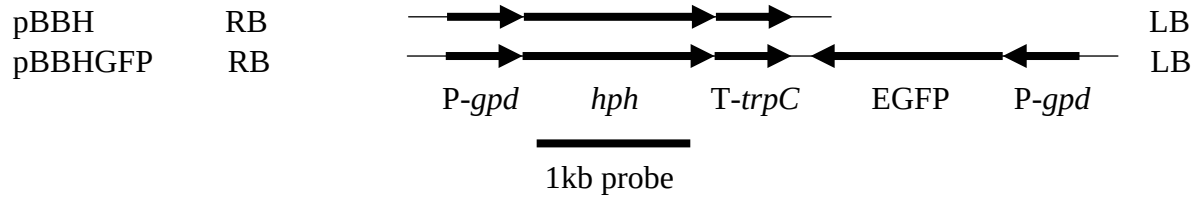
Plasmids and bacterial strains

The DH5α strain of *Escherichia coli* was used for maintenance of plasmids. Strain C58 of *A. tumefaciens* containing the Ti plasmid pGV2260 was used for maintenance of constructs and for *A. tumefaciens*-mediated transformation.

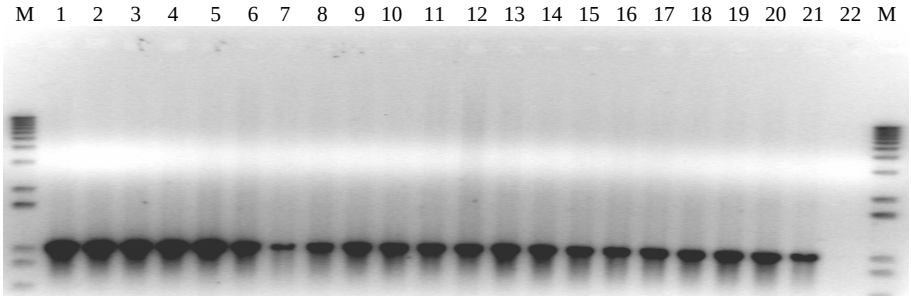
The binary vectors used for this study, pBBH and pBBHGFP, were constructed on the backbone of pBBLBRB deriving from pBBR1 (Rudy and Locht, 1992). This plasmid contains a kanamycin resistance gene as well as the left and right border sequences of *A. rhizogenes* strain A4 surrounding a multiple cloning site. A *Bgl*II-*Hin*dIII fragment of pAN7-1 (Punt et al. 1987), containing the hygromycine B resistance (*hph*) gene under the control of the *Aspergillus nidulans gpdA* promoter, was isolated and cloned between the *Bam*HI and *Hin*dIII sites of pBBLBRB to produce pBBH. A second vector named pBBHGFP was constructed by excising the enhanced green fluorescent protein (EGFP) gene with the *gpd* promoter from pCB1265 using *Pst*I and *Hin*dIII and inserting this restriction fragment at the *Pst*I-*Hin*dIII

sites in pBBH (Fig. 1A). These constructs were introduced into *A. tumefaciens* by electroporation at 1.5 kV, 200 ohms and 25 μF.

A



B



C

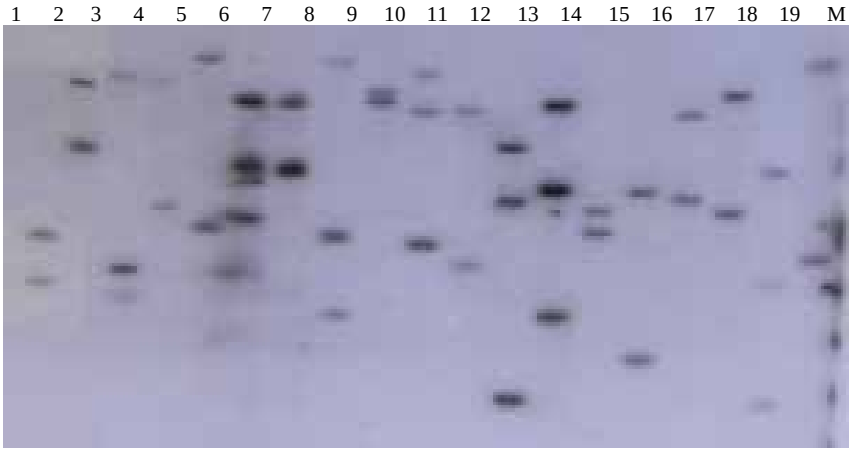


Figure 1: Analysis of *L. maculans* transformants obtained after *A. tumefaciens*-mediated transformation.

A Schematic representation of pBBH and pBBHGFP containing a 4 kb and a 6.6 kb insert, respectively. RB T-DNA right border repeat, P-gpd *Aspergillus nidulans* glyceraldehyde-3-phosphate deshydrogenase promoter, *hph* hygromycin B phosphotransferase gene, T-*trpC A. nidulans* trpC terminator, EGFP enhanced green fluorescence protein gene and LB T-DNA left border repeat

B PCR screen of 21 randomly chosen hygromycin-B resistant transformants (lanes 1-21) and the wild-type recipient isolate v23.1.3 (lane 22). M: 1Kb+ DNA ladder (Life Technologies)

C Southern blot analysis performed on 19 independent transformants. Genomic DNA was digested with *Eco*RI and probed with the 1kb *hph* internal fragment. M: sizes of marker DNA fragments (Raoul ^{TM} Marker, Q.Biogene)

Agrotransformation

Transformation of conidia of isolate v23.1.3 was essentially as described by de Groot et al. (1998). Agrobacteria were grown for 24 h in liquid LB medium containing ampicilline and rifampicin at 28°C with shaking. They were then induced by adding 2 ml of bacteria suspension to 18 ml of liquid induction medium containing acetosyringone and growing them for 6 h at 28°C with shaking. *L. maculans* conidia were collected from sporulating cultures by rubbing the surface of the colonies in 5 ml of Modified Fries medium. Conidia concentration was brought to 10^8 ml^{-1} and the suspension was allowed to germinate in liquid Fries medium by incubation for 24 h at 28°C with shaking. Two-ml of induced Agrobacteria suspension were mixed with 2 ml of germinating conidia suspension, and 200 µl of the mix were plated on cellophane deposited on agar medium containing acetosyringone. Following 60 h of growth at 20°C, the cellophane disks were transferred onto agar medium containing 200 mM cefotaxime and 200 µM hygromycin. Growth conditions were at 22°C with light and a 12-hour photoperiod. Growing colonies were transferred to minimal medium MMII containing cefotaxime and hygromycin. After sub culturing once on selective medium, individual putative transformants were induced to sporulate and monopycnidial cultures were obtained by picking conidia oozing from one pycnidium that was transferred to selective media.

Fungal DNA extraction

Mycelia from the transformants as well as wild type strain grown in liquid Fries medium for two weeks were harvested by filtration and freeze-dried, ground to a fine powder, and DNA extraction was carried out as described by Balesdent et al. (1998). As an alternative, DNA was extracted from conidia suspensions as described previously (Attard et al. 2001): one ml of each conidial suspension containing approximately 10^8 conidia per ml was put in a microtube and frozen. A tungsten carbide bead was then added and the samples were frozen again in liquid nitrogen. The grinding was performed by shaking vigourously the tubes for 2 x 1.5 min with a Mixer Mill MM 300 (Retsch) at maximum speed. DNA purification was performed using the DNeasy 96 Plant Kit (Qiagen) according to the manufacturer's instructions, using the BioRobot 300 (Qiagen).

PCR amplification

PCR amplification was carried out with a primer pair designed to amplify part of the *hph* gene (5' GCCTGAACTCACCGCGACG 3'/ 5'TTCCTTTGCCCTCGGACGAGTGC3'). PCR conditions were the following: 5 min at 94°C (first cycle); 30 s at 94°C; 15 s at 64°C; 1 min 30 at 72°C (30 cycles); 7 min at 72°C (last cycle). The same primers were used with pAN7.1 as a template to obtain a probe for use in Southern analysis.

Southern blotting

Genomic DNA (10µg) was digested with *Eco*RI and resolved on a 0.8% agarose gel. Southern blots were prepared with the Positive ^{TM} Membrane (Q.Biogen) and hybridization with the *hph* gene fragment (α-^{32}P-labelled) was performed under high stringency conditions as described by Attard et al. (2001).

Expression of EGFP

Putative EGFP-expressing conidia and hyphae were identified using a LEICA DMLB fluorescence microscope fitted with an I3 blue filter set.

Biological and phytopathological characterization of the transformants

Morphological aspect and sporulation rate of transformed isolates were firstly assessed visually following culture on V8-agar medium. Germination efficiency of conidia was measured on a few mutants or progeny by plating 50 conidia on PDA medium, with five replicates, and counting the colonies formed. Growth rate was assessed following deposition of a 10-µl droplet of 10^7 conidia suspension onto MMII agar media and measuring the diameter of the colonies at three times after growth at 20°C in the dark, with three replicates. Pathogenicity of transformants was assessed on cotyledons of oilseed rape cv. Westar as described previously (Balesdent et al. 2001), except that scoring took place every 2 days, departing from 9 days post inoculation. In the early stages of the infection, the lesion diameter was measured, whereas in the latter stage (past 14 dpi), the semi-quantitative IMASCORE rating scale was used.

Results and discussion

Transformation

Preliminary experiments showed that the growth of *L. maculans* was totally inhibited on media containing 50-100 µg ml^{-1} of hygromycin B (J. Schmit, J.P. Narcy and J. Roux, unpublished data). Such selective media were therefore chosen for the selection of resistant colonies in the transformation experiments.

A. tumefaciens strain C58 with the binary vectors pBBH or pBBHGFP was used to transform *L. maculans*. Co-cultivation of *Agrobacterium* with a conidial suspension of the *L. maculans* isolate v23.1.3 (5 x 10^7 conidia per ml) led to the formation of hygromycin B-resistant colonies approximately 14-20 days after transfer to the selective medium (data not shown).

The efficiency of transformation was the same with each binary vector and reached approximately 120 colonies per $1x10^7$ conidia. When subcultured to minimal medium amended with hygromycin B (100 µg ml^{-1}), all putative transformants maintained their Hyg^R phenotype. In a preliminary experiment, a "pilot collection" of 153 Hyg^R transformants was generated (120 obtained with pBBH and 33 obtained with pBBHGFP). Additional larger-scale collections of transformants were routinely generated by transformation with pBBH at a latter stage.

Molecular characterization of the transformants

To study whether the *hph* gene was present in the putative transformants, genomic DNA from 29 isolates transformed with pBBH was analysed by PCR. A band of the expected size was obtained with DNA from all transformants but not with DNA from untransformed *L. maculans* (Fig. 1B).

Southern blotting analysis was performed for these 29 transformants using as a probe a 1kb *hph* internal fragment (Fig. 1A). The *hph* gene probe has one *Eco*RI restriction site, corresponding to the enzyme used to digest genomic DNA. Hence, in Southern blots, two hybridising bands were expected to occur in the case of a single-copy insertion. This pattern was effectively obtained for 18 transformants (Fig. 1C). The size of the bands differed for each transformant indicating that single integration events have occurred at random loci (Fig. 1C). Two of these transformants had four hybridizing bands indicating that two copies of the *hph* gene had been inserted. Nine other transformants had a more complex pattern with three hybridizing bands. This suggests that they contain at least one copy of the *hph* gene. The additional bands could have been caused by tandem integrations or the integration of vector

sequences external to the T-DNA as this has been reported for *A. tumefaciens*-mediated transformation of *Fusarium circinatum* (Covert et al. 2001).

Mitotic and meiotic stability

Mitotic stability of the 153 transformants of the "pilot collection" was tested by subculturing three times onto V8-agar without hygromycin B, and a fourth subculture on minimal medium containing 50-100 μg ml^{-1} of hygromycin B. All the transformants retained their resistance to antibiotics through these successive serial subcultures.

To investigate the meiotic stability of integrated T-DNA copies, six transformants of isolate v23.1.3 (Mat+) that carried single copies of the T-DNA were crossed with the wild type, sexually compatible, sister isolate v23.1.2 (Table 1).

Table 1. Genetic analysis of the segregation of mutant phenotypes along with the *hph* gene in selected transformants of isolate v23.1.3.

Mutant id	Phenotype	Nb. of insertions (Southern blotting)	Cross efficiency	Hyg^R segregation	Co-segregation with phenotype	Mutation event
20	Non pathogenic	1	low	24+/23- (single insert)	yes	Tagged candidate gene
55	Highly aggressive	1	high	22+/25- (single insert)	no	Untagged mutant
76	Weakly aggressive	1	low	In progress (two inserts?)	yes ??	Tagged candidate gene ?
40	Weakly aggressive	1	high	30+/38- (single insert)	no	Untagged mutant
43	Highly sporulating	1	high	21+/29- (single insert)	no	Untagged mutant
44	Highly sporulating	2	high	43+/31- (one or two?)	no	Untagged mutant
4	Weakly sporulating	1	high	22+/21- (single insert)	no	Untagged mutant
49	Weakly sporulating	2	sterile	?	?	?

No progeny was obtained with transformant 49 despite three attempts to cross it with isolate v23.1.2. All other crosses gave rise to the development of pseudothecia and random ascospore progeny were recovered (Table 1). Segregation of the Hyg^R character in random ascospore progeny was analysed by PCR amplification to verify the presence of the *hph* gene and by transferring mycelium to selective medium to examine the expression of the *hph* gene. As expected, all colonies in which the *hph* gene was detected by PCR grew on selective medium. Moreover, whenever a single-gene control was expected following Southern analysis, resistant:sensitive progeny segregated 1:1 as expected for a single gene (Table 1). The only exception was mutant 76 for which segregation of the Hyg^R character could be indicative of a digenic control in preliminary experiments, whereas Southern blots are suggesting a single integration (Table 1).

GFP expression of transformants

After *Agrobacterium*-mediated transformation of *L. maculans* with the pBBHGFP plasmid, 20 independent transformants were examined to detect the EGFP gene expression by GFP fluorescence. Conidia and hyphae grown in-vitro were viewed using a fluorescence microscope. For the untransformed isolate, no fluorescence was detected. In contrast, all the transformants but two exhibited green fluorescence. However, there were clear-cut differences between transformed isolates in terms of intensity of fluorescence (Fig. 2). These differences of fluorescence between transformants could be due to differences either in the genomic regions where the insertion had taken place, leading to repression in the expression, or to overexpression, of the GFP or in the number of copies of T-DNA integrated. One can also suppose silencing due to multiple integrations in the fungal genome. Finally, one cross was performed between a GFP-expressing transformant and v23.1.2. A 14:9 segregation ratio was obtained for Hyg^R phenotype, indicative of a single gene insertion, and GFP expression was fully conserved in all progeny expressing the Hyg^R character, including in ascospores resulting from the cross (data not shown).

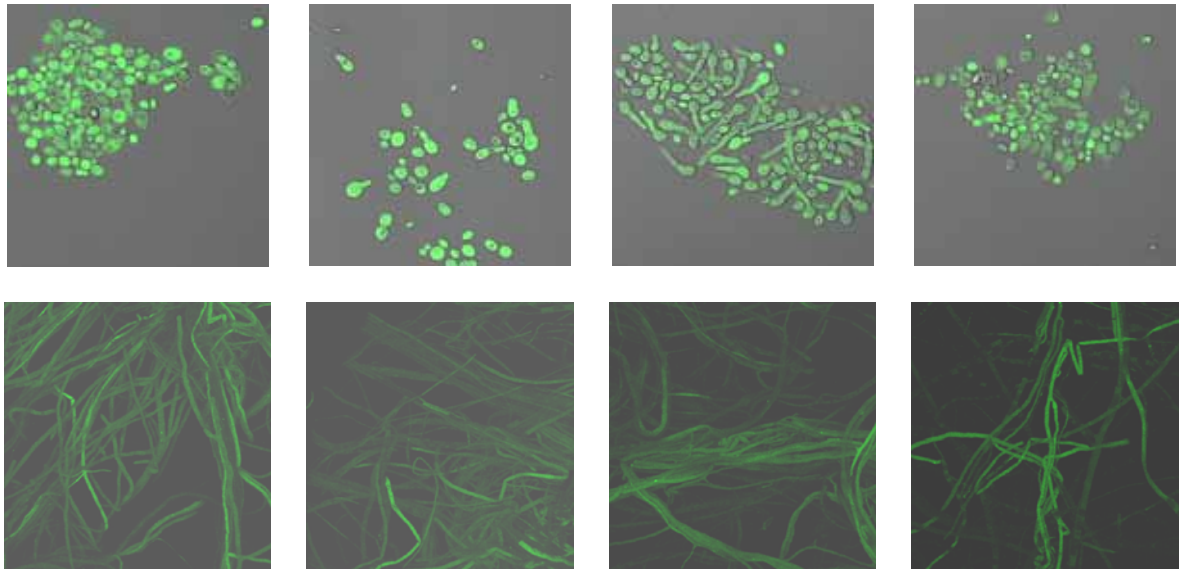


Figure 2: Confocal imaging of germinating conidia and hyphae of four randomly chosen *L. maculans* transformants expressing EGFP

Biological and phytopathological criteria

Among the biological and phytopathological criteria examined, some like sporulation intensity, colony morphology or cantitative aspects of pathogenicity (aggressiveness) were found unreliable criteria. Mainly, transformants selected for putative alteration in sporulation intensity were all found to be untagged in the progeny (Table 1), for which an extremely wide range of sporulation ability was observed between isolates.

Other criteria, in contrast, were extremely robust and included radial growth rate on agar medium (Fig. 3), germination efficiency of conidia, and virulence on cotyledons of oilseed rape (data not shown). When analysing the collections for biological criteria, only few transformants were affected in their growth rate as compared to the v23.1.3 wild-type isolate (Fig. 3). Similarly, when analysing the “pilot collection” of 153 transformants, only 4 (2.6%) were found to be altered in their pathogenicity (Table 1). Of these, one was non-pathogenic, 2 were weakly aggressive, and a last one was more aggressive than the wild-type (Table 1). Of these, one was unequivocally tagged, with the Hyg^R phenotype fully co-segregating with the

altered phenotype, whereas one weakly aggressive showed a phenotype segregation independent from that of the *hph* gene and one strongly aggressive showed loss of the phenotype in the progeny (Table 1).

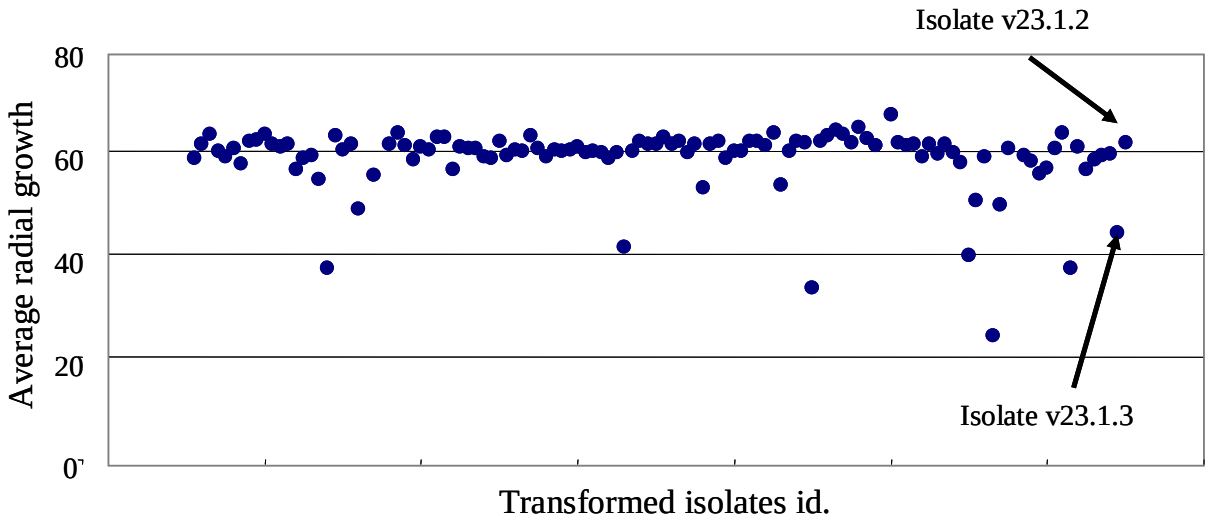


Figure 3: An example of radial growth observed on MMII agar medium (after 28 days at 20°C) for a collection of 120 isolates transformed with the pPBBH vector. The wild-type parent isolate, v23.1.3, along with the sister isolate used for segregation analysis, v23.1.2, are indicated.

Discussion

In this paper, we report for the first time the *Agrobacterium*-mediated transformation of *L. maculans*. This has been done using a new binary vector derived from a plasmid isolated from *Bordetella bronchiseptica* and the strain C58 of *Agrobacterium tumefaciens*.

To date, four strains of *Agrobacterium* (LBA1100, LBA1126, AGL0 and AGL1) have been used for the transformation of filamentous fungi (de Groot et al. 1998; Zwiers and de Waard 2001; Malonek and Meinhardt 2001; Mullins et al. 2001; Covert et al. 2001; Rho et al. 2001). All these strains including C58 share a C58 chromosomal background but they differ by the *Ti* plasmid they possess. Three of them (LBA1126, AGL0, AGL1) have been chosen because they have a hypervirulent phenotype. This is due to mutations in the *virG* locus on their *Ti* plasmid, which enables the production of high level of *vir* gene products (Hellens et al. 2000). Our work therefore allows broadening of the choice of *A. tumefaciens* strains that can be used to transform fungi.

The number of transformants obtained (120 per 10^7 conidia) is in the same range as for other filamentous fungi, e.g., 5 resistant colonies per 10^7 spores in *Agaricus bisporus* and up to 5000-10000 transformants per 10^7 spores in *Magnaporthe grisea*. An interesting point is that a high number of the 29 transformants analysed (approximately 60%) have a single-copy insertion. This percentage is close to those found in the case of *Fusarium oxysporum* and *M. grisea* (Mullins et al. 2001; Rho et al. 2001). However, in contrast to the results obtained with these two fungi, we did not find transformants with more than two copies of T-DNA. This difference might be attributed to the conditions used during experiments, i.e., the co-cultivation time with which *A. tumefaciens* cells and fungal conidia were incubated.

Previous studies on transformants of *L. maculans* obtained by PEG-mediated transformation have shown that the integration of the *hph* gene is stable through mitosis

(Farman and Oliver 1988). Our work not only confirmed the mitotic stability of the insert, but also indicates that the stability of the gene is maintained through meiosis. During meiosis in *L. maculans*, novel chromosomal length polymorphisms produced by reciprocal recombination between parental homologous chromosomes of unequal sizes are frequently observed (Plummer and Howlett 1995, Leclair et al. 1996). In our case, these possible rearrangements of chromosomal DNA have not affected the stability of the resistance marker for the crosses investigated.

On the other hand, our segregation data indicates that the mutations for some analysed mutants were not due to the plasmid integration event, but rather to alterations linked with the transformation process or to the natural variation associated with asexual multiplication in this fungal species. In this respect, the range of variation in some biological criteria, such as sporulation intensity, is so wide that there is a complete independence between this criteria and insertion of the *hph* gene. In contrast, more robust criteria, such as pathogenicity, can allow us to evaluate the efficiency of the transformation process to generate mutants which are actually tagged by agrotransformation. At the moment, the efficiency of the *Agrobacterium*-mediated transformation technique to tag mutants is not known because it is a quite recent methodology applied to fungi, and no screening of large collections of mutants has been reported. Mainly, there has not been any genetic validation of co-segregation between the insertion and the altered phenotype performed to date in other fungal species. This paper is therefore the first report evaluating the efficiency of the tagging process linked with the *Agrobacterium*-mediated transformation in fungi. Even though only regarding 4 pathogenicity mutants, our data therefore suggest that two mutants (50%) were not tagged whereas 1 or 2 are indeed tagged by the *hph* gene. If these data are confirmed on larger collections of transformant this will indicate a quite efficient protocol to generate large collection of pathogenicity mutants tagged in genes of interest.

Finally, we show that the eGFP gene is expressed both in conidia, ascospores and mycelium of *L. maculans* by detecting GFP fluorescence. EGFP has been used as a cytological marker to follow the progress of *L. maculans* during infection of *B. napus* and *B. juncea* (Sexton and Howlett 2001). The possession of GFP-expressing isolates will allow experiments to be carried out into the survival, growth, epidemiology and host colonisation of *L. maculans*.

In summary, we demonstrate that the use of *A. tumefaciens*-mediated DNA transfer is a simple and efficient method for transforming *L. maculans*. As a high number of transformants can be obtained rapidly, this procedure can be used to generate a large library of fungal transformants, which can be subsequently used to screen the mutants that exhibit loss of (or reduced) virulence. The identification of genes that are important for pathogenicity will be facilitated by the fact that a high percentage of transformants have a single-copy insertion.

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Resistance of Brassicas towards *Xanthomonas campestris*

Screening of *B. napus* with *Xanthomonas campestris* pv. *campestris* and *Leptosphaeria maculans*

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Abstract: Ninety accessions of *Brassica napus* were screened against two isolates of *Xanthomonas campestris* pv. c*ampestris*, (Xcc524, race 4 and Xcc512, race 1), using inoculation by injection of the 10^8 cfu/ml bacterial suspension directly into the leaf's veins of 3-4 true leaves plants. All the ninety accessions were found to be very susceptible to isolate Xcc512. Twenty-three accessions presented more than 25% of the plants resistant to isolate Xcc524. The most resistant accessions were CGN14113, ISA156, ISA78, BRS0054 and ISA666. These accessions were further screened against other nine Portuguese and foreign isolates (Xcc513, race 0; Xcc545=PHW117, race 1; Xcc459=2D520, race 2; and Xcc510, Xcc516, Xcc501, Xcc530, Xcc547=HRI1279a, and Xcc550=Dickson171 all race 4). All the accessions, except CGN14113 that presented moderate levels of resistance to Xcc516, were susceptible to isolates Xcc513, Xcc545, Xcc459, Xcc510 and Xcc516. GGN14113 was also the only accession presenting resistance to isolates Xcc501, Xcc530, Xcc547 and Xcc550.

The twenty-three resistant accessions to *Xcc* were further screened at the cotyledons against four isolates of *Leptosphaeria maculans* (BBA62908, PG2-A3; T12aD34, PG4-A1; PT01, PG4-A1; and V11.1.1, PG4-A1). Only two accessions ISA666 and NU51661/2 were resistant to the four isolates. Accessions ISA302 and CGN14113, ISA156, ISA78, BRS0054, ISA381, and ISA305 presented respectively resistance and moderate resistance to isolate BBA62908. Accession BRS0085 was resistant to isolate V11.1.1 and moderately resistant to the other isolates and accessions ISA 78 and BRS0061 were moderately resistant to isolates T12aD34 and PT01 and V11.1.1, respectively. All the other accessions were susceptible to all the isolates.

ISA666 and NU51661/2, respectively with 54% and 42% of resistant plants to isolate Xcc524 and resistant to all the four isolates of *L. maculans* studied, are new interesting sources of resistance for *B. napus* breeding.

The exploitation of genetic resources of *Brassica juncea* for resistance to *Xanthomonas campestris* pv. c*ampestris*

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Abstract: Fifty accessions of *Brassica juncea* were screened against four isolates of *Xanthomonas campestris* pv. c*ampestris*, Xcc524 (race 4), Xcc512 (race 1), Xcc459 (=2D520, race 2) and Xcc521 (race 0), using inoculation by injection of the 10^8 cfu/ml bacterial suspension directly into the leaf's veins of 3-4 true leaves plants. It was found that 92%, 90%, 16% and 0% of the accessions were respectively resistant to those isolates. The most resistant accessions were the lines "HRI7677/1" and "HRI7677/3", and the cultivars "Leaf Mustard", "West Lake", "Late Head" that were resistant to all those isolates, except the race 0 Xcc521 isolate.

Twelve selected mustards were screened at the 3-4 true leaves, using the same technique, with fourteen isolates of *X. campestris* pv. *campestris*, including isolates races 0, 1, 2 and 4 isolated from *B. oleracea* and *B. rapa*. In general the 12 accessions were resistant to isolates races 1 and 4 and susceptible to isolates races 0 and 2. The isolates isolated from *B. rapa* were the most virulent in the accessions tested. Only accessions "HRI7677/1" and "HRI7677/3" were high resistant to two isolates race 0 (Xcc513 and Xcc523) and to isolate race 2 Xcc549 which is new since the few mustards screened until now in previous studies were only considered resistant to isolates races 1, 3 and 4. This study confirms the high potential of mustards as sources of resistance of black rot and has identified two new accessions with a high valuable resistance.

ENTOMOLOGISTS SECTION

Abundance and spatial distribution of pests in OSR crops

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Effects of a turnip rape trap crop on the spatial distribution of *Meligethes aeneus* and *Ceutorhynchus assimilis* in oilseed rape

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Abstract: Alternative control strategies for insect pests of oilseed rape (*Brassica napus*) are needed to help reduce the current reliance on insecticide treatments. We investigated the effect of a turnip rape (*Brassica rapa*) trap crop border on the abundance and spatial distribution of two major oilseed rape pests, the pollen beetle (*Meligethes aeneus*) and the cabbage seed weevil (*Ceutorhynchus assimilis*) in oilseed rape plots. Pest abundance was recorded throughout the growing season at spatially referenced sampling points evenly arranged within the plots. Growth stage of the plants in the plot centres and borders were also assessed. The turnip rape developed more quickly than the oilseed rape. Pollen beetle abundance was greater overall in the plot with a turnip rape border than in the control plot. However, beetles were retained in the turnip rape border and consequently the population in the oilseed rape plot centre was lower than in the plot without a turnip rape trap crop. This effect lasted throughout the pollen beetle damage-susceptible stage of oilseed rape (green-yellow bud) and until the oilseed rape was in late flower and the turnip rape trap crop was in pod. Seed weevil infestation was low and the turnip rape trap crop had little effect on weevil distribution. The usefulness of this trap-cropping strategy for control of the pollen beetle and seed weevil is discussed.

Key words: *Brassica rapa*, *Brassica napus*, trap cropping, pollen beetle, cabbage seed weevil

Introduction

Insecticide application is still an integral part of insect pest management in oilseed rape (*Brassica napus*). However, alternative control strategies are ever-more required, due to the negative effects of these chemicals on non-target organisms - including beneficial species, increasing pest resistance and concerns over spray-operator safety. Trap cropping systems offer one such alternative. They work on the principle that most pests show preferences for certain plant species, cultivars, or growth stages over others. These preferred plants can be used as 'trap crop' stands that attract the colonising pests away from the main crop, concentrating them on the trap plants where they can be more effectively controlled (reviewed in Hokkanen, 1991).

The pollen beetle (*Meligethes aeneus*) and the cabbage seed weevil (*Ceutorhynchus assimilis*) are two major pests of oilseed rape, damaging the buds and pods, respectively. It has been demonstrated that turnip rape (*Brassica rapa*) is more preferred by these pests and has potential for use as a trap crop to reduce damage to oilseed rape (Hokkanen *et al.*, 1986; Buechi, 1990; Nerad and Vašák, 2000; Cook *et al.*, 2002, 2003). Hokkanen *et al.* (1986), Buechi (1990) and Cook *et al.* (2002) all proposed that the turnip rape trap crop would be effective if planted as a border around the perimeter of the crop. However, the effect of this system on the spatial and temporal distributions of the pests has never been studied. Furthermore, its effectiveness in reducing pest damage in the protected areas has not been clearly demonstrated.

We conducted a small experiment in field conditions to investigate how a turnip rape trap crop border influences the infestation and subsequent spatio-temporal distribution of pests, as well as the damage they cause. We did this to address the concern that the turnip rape trap crop could actually increase infestation and damage levels to oilseed rape by attracting more pests into the vicinity than would otherwise be present if the turnip rape was absent.

Materials and methods

Experimental plots

Two plots of spring oilseed rape (*cv*. Canyon), were grown on Rothamsted Farm, Harpenden, Hertfordshire, UK. The plots (24 m x 24 m) were surrounded with either a 3 m-wide border of turnip rape (*cv*. Agena) (OSR/TR) or by an equal-sized extension of the main crop as a control (OSR/OSR). Total plot area was 30 m x 30 m. Each plot was sub-divided into 36 spatially referenced 'sample points' (5 m x 5 m), evenly distributed across the plot. The outermost 20 sample points along the edges of the plot were referred to as the plot 'border' and the innermost 16 sample points in the middle of the plot were referred to as the 'centre', encompassing the turnip rape trap crop/oilseed rape control border and oilseed rape plot centre, respectively. The plots were monitored throughout the 2002 season from the green bud stage until podding; plant growth stages of the oilseed rape and turnip rape plants in the plot centres and borders were recorded using the system of Lancashire *et al*. (1991), and adult pest abundance/distribution and pest damage was also assessed.

Adult pest abundance & distribution

To assess the effect of the turnip rape trap crop on adult pollen beetle and seed weevil abundance and distribution, the two plots were sampled for adult infestation every 3-4 days from the green bud growth stage (GS 50-53, mid-June) until podding (GS 80, mid- August). At each sample point, five plants were selected at random and the number of adult pollen beetles and seed weevils on each plant was assessed by beating the plant over a white plastic tray.

The mean number of pollen beetles and seed weevils per plant on the OSR/TR plot and the OSR/OSR plot were plotted for each sample date. Contour maps of the mean numbers of pests per plant at each sample point on both plots were produced on four dates, so that the effect of the turnip rape trap crop on the initial infestation and subsequent changes in abundance and distribution could be assessed as the plants developed (Surfer 7, Golden Software Inc., Colorado, U.S.A.).

Pollen beetle damage

To compare the levels of pollen beetle damage between the two plots, a sample of buds was taken from each plot. The terminal racemes were taken from five plants selected at random from each of the sample points in the two plots. In the laboratory, the number of buds per raceme was recorded. The buds were dissected and the number accepted for pollen beetle oviposition (i.e. those that contained eggs and/or larvae) was recorded. The proportion of accepted buds at each sample point was calculated, and used as a measure of damage. Damage levels in the centres of OSR/TR and OSR/OSR plots were compared using a two-sample binomial test (two-sided, using normal approximation, GenStat, Version 7, VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Damage occurring in the borders of the two plots was compared in the same manner. Damage assessments in the plot centres and borders for both plots were mapped on a spatial scale using Surfer software (as above).

Seed weevil damage

To compare the levels of seed weevil damage between the two plots, ten pods were selected at random from each of the 36 sample points in both plots. In the laboratory, the pods were dissected and the proportion that contained eggs and/or larvae was used as a measure of damage. Damage levels in the centres and borders of OSR/TR and OSR/OSR plots were each compared as described above for pollen beetle damage.

Results

Adult pest abundance & distribution

The turnip rape plants developed more quickly than the oilseed rape plants, and flowered about 10 days before the oilseed rape (Table 1).

Table 1. Growth stage (Lancashire *et al*., 1991) of spring oilseed rape and turnip rape plants in plots sampled throughout the flowering phase in 2002.

Sample	Date	Growth stage oilseed rape	Growth stage Turnip rape
1	25 June	50, buds enclosed	51-53, buds extending
2	28 June	51-53, buds extending	55-59, buds extended-yellow bud
3	2 July	52-55, buds extended	59-61, yellow bud-10% flowering
4	8 July	55-59, yellow bud	61-63, 10-30% flowering
5	12 July	59-61, 10% flowering	65, 50% flowering
6	15 July	63, 30% flowering	67, flowering declining
7	18 July	65, 50% flowering	67-69 end of flowering
8	22 July	67, flowering declining	71-73, 10-30% pods full size
9	25 July	67-69 end of flowering	73-75, 30-50% pods full size
10	29 July	71-73, 10-30% pods full size	75-77, 50-70% pods full size
11	2 Aug.	73-75, 30-50% pods full size	80, pods ripening,
12	5 Aug.	75-77, 50-70% pods full size	81-83, 10-30% pods ripe
13	12 Aug.	77-80, 70% pods full size,	83-85, 30-50% pods ripe

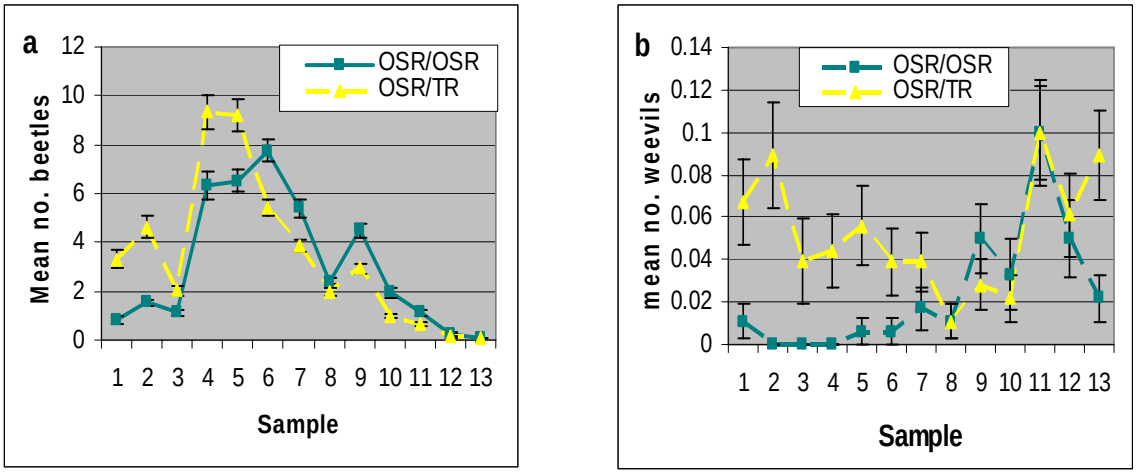

Figure 1. Mean (±SE) pollen beetles (a) and seed weevils (b) per plant on oilseed rape plots with (OSR/TR) and without (OSR/OSR) a turnip rape trap cop border on 13 sample dates. The dates of each sample and growth stages of the oilseed rape and turnip rape plants are shown in Table 1.

Pollen beetle abundance peaked between samples 4-6, when the oilseed rape was in yellow bud and the turnip rape was in early flower (Figure 1a). Abundance on the plot with the turnip rape trap crop border (OSR/TR) was initially greater than on the plot without it (OSR/OSR) (Figure 1a); thereafter, there was little difference in the number of beetles between the two plots. From the contour plots (Figure 2), it can be seen that, although pollen beetle abundance was initially greater in the OSR/TR plot, their distribution was concentrated in the trap crop areas and the abundance of beetles in the oilseed rape plot centre was lower than in the OSR/OSR plot without the trap crop (Figure 2 a & b). As the oilseed rape came into flower, the distribution between plot centres and borders in the two plots became more equal (Figure 2 c & d).

Seed weevil abundance peaked at sample 11 (2 August), when the oilseed rape was in early pod and turnip rape pods were beginning to ripen, although abundance was low throughout the trial (Figure 1b). Numbers were initially greater in the OSR/TR plot than the OSR/OSR plot until flowering of turnip rape declined (Figure 1b). Until 12 July the weevils were most abundant in the turnip rape trap crop borders (data not shown). Thereafter, the turnip rape trap crop had little effect on the distribution of seed weevils (Figure 3).

Pollen beetle damage

Pollen beetle damage to buds sampled on 15 July (at the peak adult infestation) differed between the two plots. The control plot was more heavily damaged than the plot with the trap crop, particularly in the plot centre (Figure 4). The proportions of oilseed rape buds damaged by pollen beetles in the centres of the plots were 20.5% and 15.8% for OSR/OSR and OSR/TR, respectively. These proportions differed significantly ($P<0.001$). In the turnip rape trap crop border, 8.4% buds were damaged and 21.7% buds were damaged in the oilseed rape control border. These proportions also differed significantly ($P<0.001$).

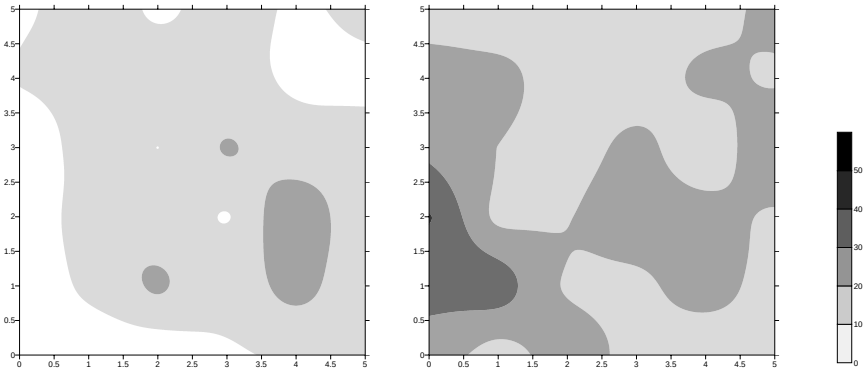

Figure 4. Contour maps showing the distribution and abundance of pollen beetle damage (% buds sampled on 15 July 2002 that were accepted for oviposition) on plots of oilseed rape with (a) and without (b) a turnip rape trap crop border.

Seed weevil damage

Seed weevil damage to pods sampled on 12 August (at the peak of adult infestation) was low, and there appeared to be little difference between the amount or distribution of damage between the two plots (contour maps not shown). The proportions of pods damaged in the centres of the plots were 3.1% and 3.8% for the OSR/TR plot and OSR/OSR plot, respectively. In the turnip rape trap crop border, 2.2% pods were damaged and 3.0% pods were damaged in the oilseed rape control border. These proportions did not differ significantly ($P=0.759$ and $P=0.606$, respectively).

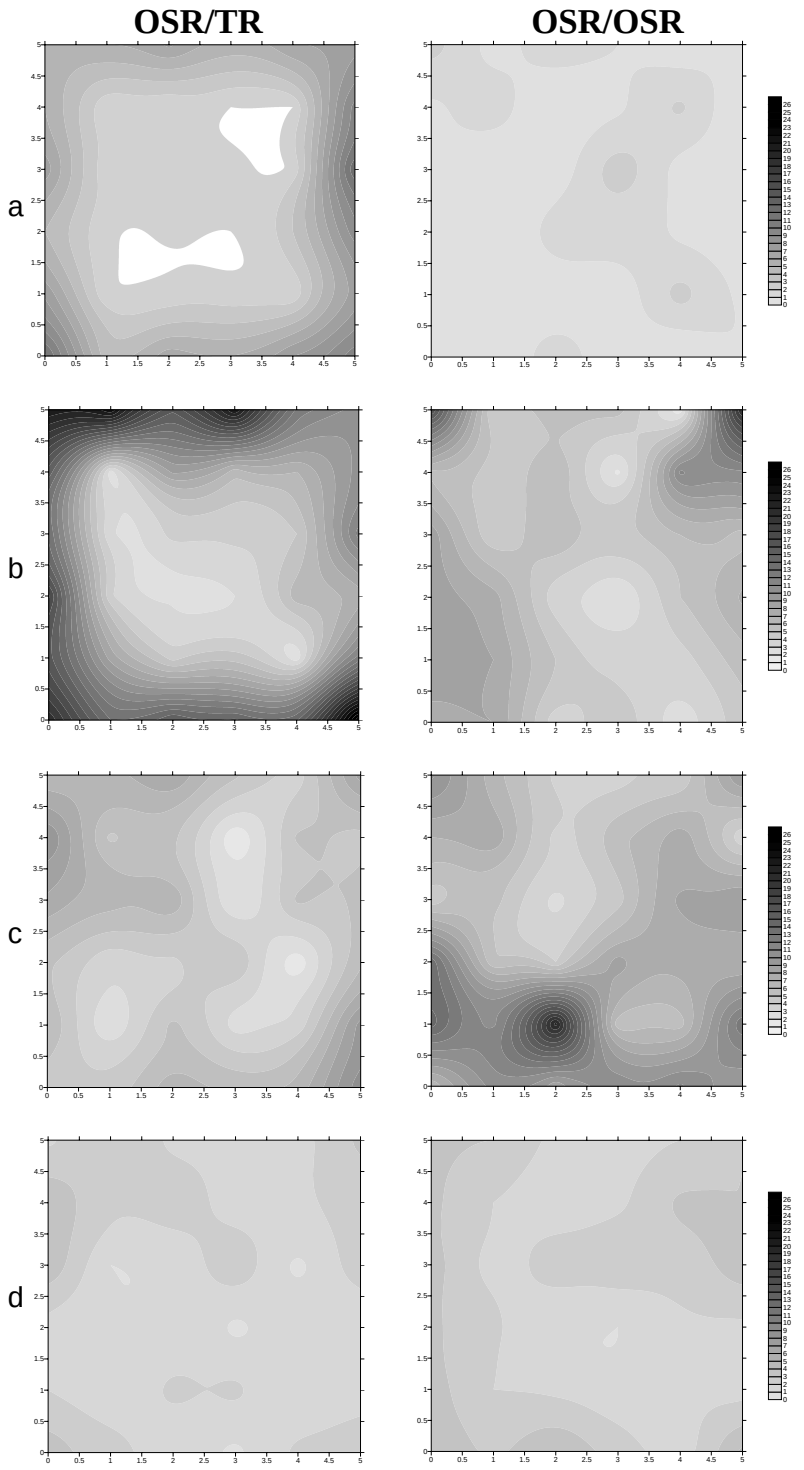

Figure 2. Contour maps showing the distribution and abundance of adult pollen beetles on plots of oilseed rape with and without a turnip rape trap crop border (OSR/TR and OSR/OSR, respectively) on four dates in 2002: (a) 25 June, (b) 8 July, (c) 15 July, (d) 22 July. Plant growth stages of the oilseed rape and turnip rape plants on these dates are shown in Table 1.

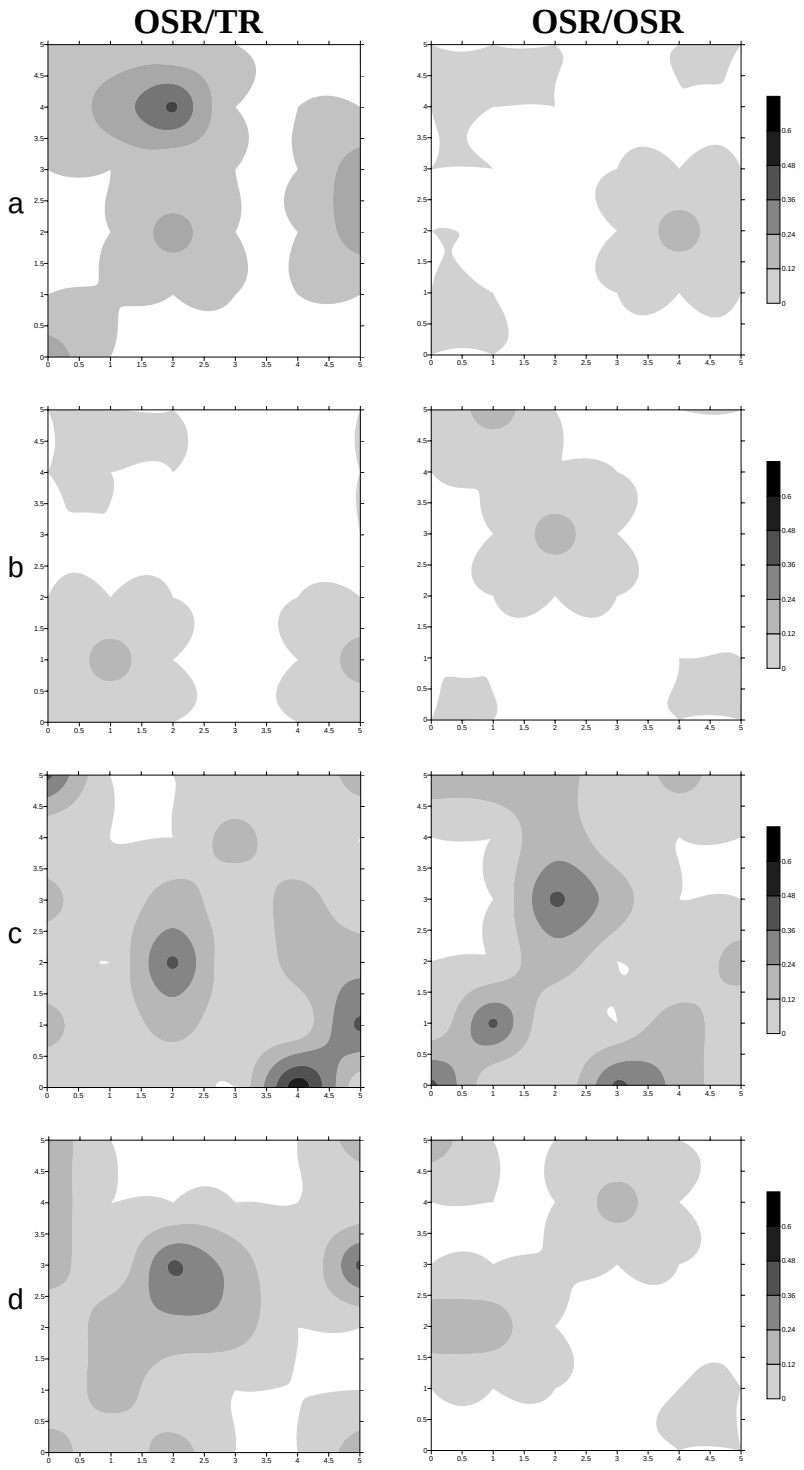

Figure 3. Contour maps showing the distribution and abundance of adult seed weevils on plots of oilseed rape with and without a turnip rape trap crop border (OSR/TR and OSR/OSR, respectively) on four dates in 2002: (a) 15 July, (b) 22 July, (c) 2 August, (d) 12 August. Table 1 shows plant growth stages of the oilseed rape and turnip rape plants on these dates.

Discussion

Although previous studies have described the spatio-temporal distributions of insect pests in oilseed rape (e.g. Free and Williams, 1979; Murchie *et al.*, 1999; Ferguson *et al.*, 2000, 2003), this is the first to explore how these factors are affected by a turnip rape trap crop. Pollen beetles infested plots from their edges at the early bud stage, as reported previously (Free and Williams, 1979; Attah and Lawton, 1984). In the presence of the earlier-maturing turnip rape trap crop border, however, beetles were even more edge-distributed than in its absence, and remained so until the oilseed rape came into flower. In the plot without the turnip rape border, the beetles distributed relatively evenly across the whole plot, confirming observations in oilseed rape fields (Free and Williams, 1979; Attah and Lawton, 1984). The population decreased in both plots as the plants ceased flowering and the beetles emigrated to find new resources. Seed weevil infestation has also been reported to occur from the crop edges (e.g. Free & Williams, 1979; Murchie *et al.*, 1999; Ferguson *et al.*, 2000). However, we did not see strong evidence of this, possibly because the populations were low in our study, or because the infestation patterns of winter and spring oilseed rape vary. The turnip rape trap crop did not thereafter appear to influence the distribution or abundance of the weevils.

This work confirms earlier studies which suggest that a turnip rape trap crop border has the potential to protect oilseed rape from pollen beetle infestation (Hokkanen *et al.*, 1986; Buechi, 1990; Nerad and Vašák, 2000; Cook *et al.*, 2002, 2003). Even though the total abundance of beetles was greater in the plot with the trap crop, the beetles were concentrated in the turnip rape and were maintained there past the oilseed rape damage-susceptible stage of green-yellow bud. Throughout the susceptible period, the abundance of beetles in the protected oilseed rape plot was lower than in the unprotected plot and was generally below spray-threshold levels. Furthermore, damage to buds in the plot centres was significantly lower in the turnip rape-protected plot than in the control. This may lead to a yield benefit, and will be investigated in future studies.

The turnip rape trap crop system was less efficient in preventing seed weevil damage than pollen beetle damage. Weevils were initially more abundant on the turnip rape trap crop borders than on the oilseed rape plants. This finding is consistent with previous reports which suggest that the seed weevil prefers turnip rape over oilseed rape (Buechi, 1990; Nerad and Vašák, 2000; Cook *et al.*, 2002, 2003), however, during early podding (the damage-susceptible stage of oilseed rape for the seed weevil), the weevils were no longer maintained in the trap crop area, and the trap crop did not affect the amount or distribution of damage in the crop. Beuchi (1990) and Cook (2002, 2003) suggested that a turnip rape trap crop without insecticides may not be able to control the seed weevil. Our study confirms these reports. However, if insecticide was applied to the turnip rape trap crop in the early stages, before flowering of the oilseed rape, then the population of weevils on the trap crop would be removed before their migration onto the central oilseed rape areas. This approach needs to be further investigated, but has the potential to control both pollen beetles and seed weevils while reducing substantially the area of crop that needs to be sprayed. Savings in insecticide costs could be expected, as well as ecological benefits; non-target organisms would remain unaffected in the unsprayed oilseed rape crop, leaving predators and parasitoids unharmed and able to help control those pests that escape insecticide control. The effect of a turnip rape trap crop in augmenting biocontrol of pests of oilseed rape by their parasitoids will be investigated in future studies.

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Spatial dynamics of pollen beetles (*Meligethes aeneus*) in relation to inflorescence growth stage within a simulated trap crop system for oilseed rape

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Abstract: The use of trap crops in the management of pollen beetles (*Meligethes aeneus* Fabricius) on oilseed rape has potential to reduce applications of pesticides. One strategy uses a trap crop of attractive early-flowering plants to protect the more susceptible late-bud stage of the main crop. Here we use semi-field arrays of potted oilseed rape plants to investigate this strategy, observing the development of distributions of pollen beetles in a simulated trap crop system. In two experiments, pollen beetle populations were counted on every plant, and related to numbers of racemes in bud and racemes in flower to assess responses to host plant resources and cues. Inflorescence growth stage characteristics were shown to be important in determining the spatial distributions of pollen beetles. The beetles were usually spatially associated with plants with more racemes in bud and/or in flower. The trap crop maintained a significant edge distribution of pollen beetles for 7-10 days. While flowering racemes provide strong cues for immigrating pollen beetles, the abundance of buds is probably a more important determinant of residence time on plants. This highlights the importance of establishing trap crops that are both attractive and provide resources that retain the pest.

Key words: *Meligethes aeneus*; *Brassica napus*; pollen beetle; oilseed rape; trap crop; growth stage; spatial dynamics.

Introduction

The pollen beetle (*Meligethes aeneus* Fabricius) is one of the major pests of oilseed rape (OSR) (*Brassica napus* L.) in Europe (Alford et al., 2003). Pollen beetles are conventionally controlled using pesticides (Walters et al., 2003). However, pesticide use in the crop is associated with greater variable costs (Lunn et al., 2001), pesticide resistance (Hansen, 2003) and harmful effects on non-target organisms (Greig-Smith, 1990). Alternative integrated pest management strategies for the crop are actively being sought (Williams, 2004).

Some of these alternative strategies, for example, push-pull strategies (Miller and Cowles, 1990) and the use of trap crops (Hokkanen et al., 1986; Cook et al., 2002b), aim to alter the spatial distributions of the pest on the crop. The development of successful strategies benefits from an understanding of how the pest locates and moves to the crop in relation to host plant and environmental factors, and, once there, how it moves within the crop. If a pest can be concentrated in a trap crop there is potential to minimise chemical inputs by treating only the trap crop with pesticide (Hokkanen, 1991; Cook et al., 2002b).

Pollen beetles are typically associated with the inflorescence growth stages of OSR. They can attain serious pest status if they infest early- and late-bud stages of winter OSR (Walters et al., 2003) and are especially attracted to plants at early-flowering growth stages of OSR (Free and Williams, 1978). Differential growth stage between host plants has potential to control pollen beetles if used within a trap crop system: Hokkanen et al. (1986) reported that in Finland a trap crop of attractive early flowering plants could be used to protect a main crop

of spring oilseed rape when at the more susceptible bud stage. Pollen beetles can migrate to crops over several weeks during the inflorescence growth stages of the crop (Free and Williams, 1978) but numbers of flowers and buds on OSR plants are continuously changing (Lancashire et al., 1991) and it is unclear how relative changes in the resources offered by trap crop and main crop plants influence the maintenance of trap crop effectiveness. The development of trap crop strategies would benefit from a better understanding of how pollen beetles respond to host plant inflorescence growth stage.

The objective of this study was to observe the development of distributions of pollen beetles in a simulated trap crop system where growth stage alone was used to manipulate the pest. The effects on pollen beetle spatial dynamics of daily changes in the relative strengths of cues from flowers and buds in trap crop and main crop plants were assessed and discussed in relation to the optimum characteristics of trap crop plants. Studying the relationship between host plant cues and pollen beetle distribution under full-scale field conditions is impractical. Instead, we assessed the natural infestation of pollen beetles on each plant of semi-field-scale arrays of oilseed rape plants arranged in pairs of plots. One plot of each pair had a simulated trap crop with an outer row of plants at early flowering stage intended to protect more susceptible inner plants at late bud stage, similar to the system used to control pollen beetles in Finland (Hokkanen et al., 1986). The other plot simulated a standard crop with all plants at the same late bud growth stage at the start of the experiment. The daily development of spatio-temporal patterns of plot infestation by pollen beetles were compared between plots and related to the growth stage of individual plants to assess the importance of cues from buds and flowers in determining distributions of pollen beetles and in maintaining the effectiveness of the trap crop.

Materials and methods

Experimental design

Potted oilseed rape (*Brassica napus* L.) plants were arranged as two square plots (test and control) 40 m apart within an open and level field at Rothamsted Farm, Harpenden, UK. The two plots were placed along an axis at right angles to the prevailing wind to minimise interplot anemotactic interference.

Each plot consisted of 100 glasshouse-grown potted plants (cv. Aries) in a 10 x 10 lattice, spaced 1 m apart; adjacent plants did not touch to ensure that any interplant movement of pollen beetles was by flight. The plants were irrigated throughout the experiment using drip irrigation to each pot. The experiment was conducted twice: in 2001, plants were placed out on 27 May and observed until 7 June; in 2002, they were placed out on 1 June and observed until 14 June.

The control plot simulated a standard field crop with all plants at a similar growth stage (late-bud stage, GS 55-60 (Lancashire et al., 1991)) at the start of the experiment. The test plot simulated a crop with a trap crop border: at the start of the experiment the outer rows of plants on all fours sides were at a later stage of growth (early-flowering stage, GS 61-63) than the inner plants which were at the same growth stage as on the control plot. Plot position was reversed between years to allow for positional bias.

Data collection

The number of racemes with flower buds only (‘racemes in bud’) and the number of racemes with flower buds and flowers (‘racemes in flower’) were counted on each plant daily in 2001 and every 2-4 days in 2002. Each year the total numbers of pollen beetles on each plant were recorded daily, starting at 09.00 h.

Data analyses

To determine the strength of any tendency to edge distribution (edge effect) or centre distributions in the plant growth variables, in pollen beetle counts or in daily changes in these variables, a randomisation test was used. This test was based on that developed by Murchie et al. (1999) and used Genstat 6 software (Genstat, Release 6.1, Sixth Edition for Windows, VSN International Ltd, Oxford, UK). The null hypothesis was that the counts were distributed randomly. To test for edge distributions, the distance to the nearest outer plant was calculated for all the plants in a plot. This distance was multiplied by the count (or change of counts) for each plant and summed to give an overall measure of the tendency of counts to be distributed away from the edge. Finally, counts were randomly permuted 200 times amongst the 100 plants and the same calculations applied to provide a frequency distribution against which the observed value could be tested. To calculate the tendency to be centre distributed, this procedure was repeated, this time calculating the minimum distance to the centre four plants of the plot. These plants were chosen to represent the centre as they were furthest away from the edge and so least likely to be affected by edge effects. When calculating the change in the counts of pollen beetles per plant from one day to the next, the first day's count was subtracted from the second day's count and 150 was added to ensure that all resultant scores were positive.

Spatial distributions of plant growth variables and of pollen beetles were compared using SADIE (Spatial Analysis by Distance IndicEs; (Perry, 1998a; Perry, 1998b)). For each distribution SADIE first calculates the spatial characteristics of the observed arrangements by comparing them to randomised permutations of the same counts amongst the sample units (plants); a set of local clustering indices is derived which indicate whether each sample unit falls within a cluster or a gap in the distribution of counts.

The overall spatial association index X (Chi) is calculated based on the similarity of the local clustering indices from two distributions (Perry, 1998a). Values of X are > 0 for distributions which are associated, around zero for distributions positioned at random with respect to one another, and < 0 for distributions which are dissociated.

Results

Distributions and dynamics of plant growth variables

On the test plots, racemes in flower showed significant edge distributions throughout both experiments (Fig. 1). Racemes in bud on the test plots showed edge distribution at the start of the experiments but they became increasingly centre distributed in the course of both experiments (Fig. 1). On the control plots in both years no centre or edge distribution was observed for either racemes in bud or racemes in flower.

Distribution and dynamics of pollen beetles

Substantial populations of pollen beetles migrated to and became established on both plots in both years (Fig. 2), and changing patterns of distribution were observed throughout both experiments.

Pollen beetles were significantly edge distributed on the test plots on all days except the final three in 2001 and the final two in 2002 (Fig. 3); the edge distribution was most marked on the first day in both years. On the control plots pollen beetles were significantly edge distributed once only, on day 10 in 2002 (Fig. 3). On most days in 2001 there were fewer pollen beetles on the inner 64 plants of the control plot than on the inner 64 plants of the test plot (Fig. 2). By contrast, in 2002, there were always more pollen beetles on the inner plants of the control plot than on those of the test plot (Fig. 2).

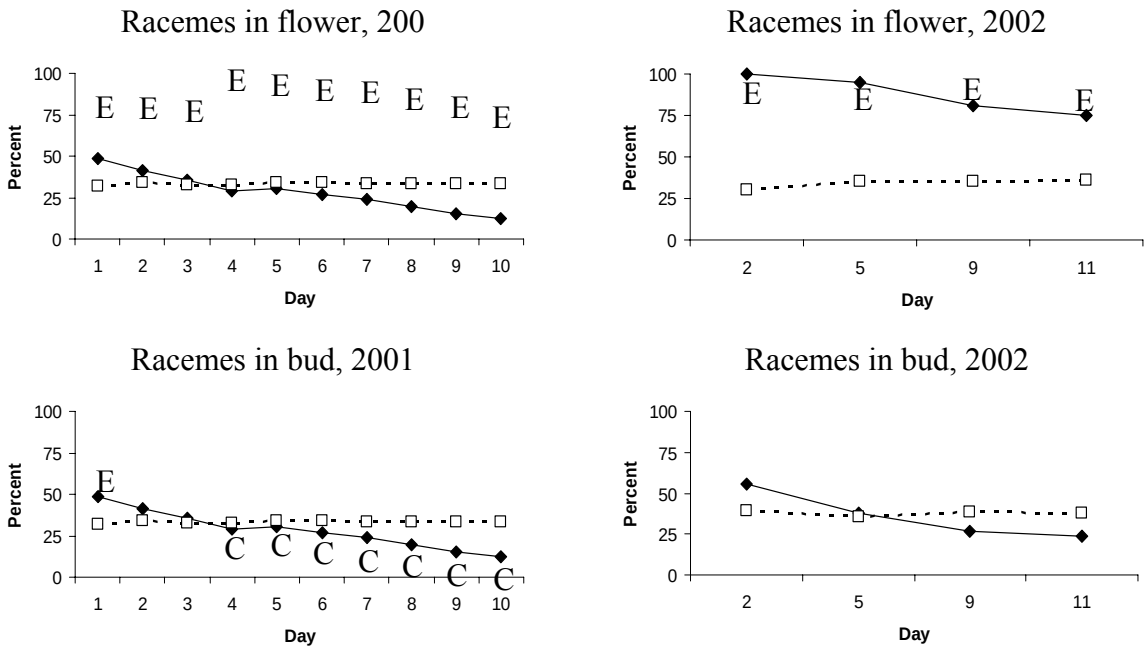

Figure 1. Percentages of total racemes in flower and total racemes in bud which were on the outer rows of the test and control plots during 2001 and 2002. —◆—, test plot; --□--, control plot. 'E', significant edge distribution on the test plot ($P < 0.01$). 'C', significant centre distribution on the test plot ($P < 0.01$).

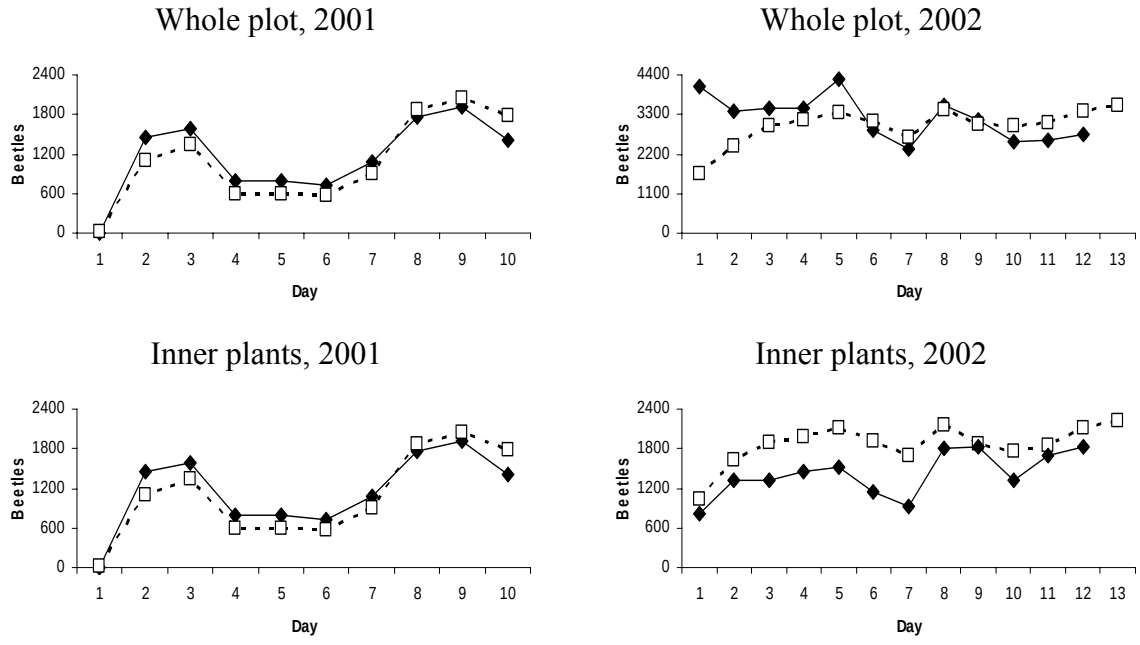

Figure 2. Total numbers of pollen beetles on the whole plot and on the inner 64 plants only, in 2001 and 2002. —◆—, test plot; -□-, control plot.

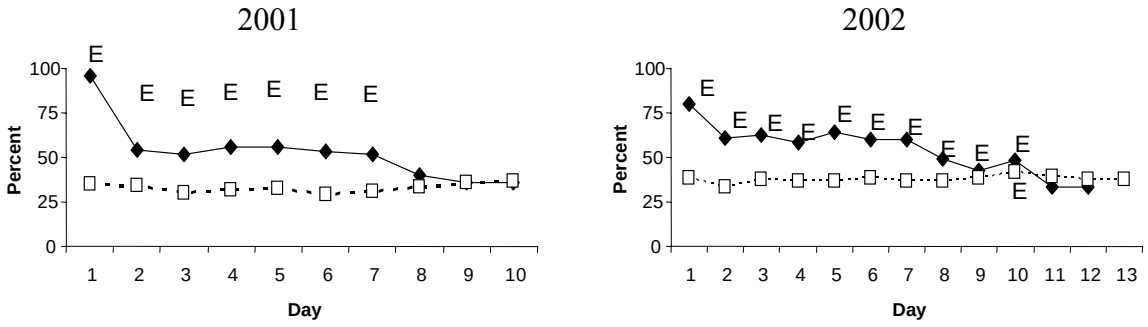

Figure 3. Percentages of pollen beetles on the outer rows of plants on the test and control plots in 2001 and 2002. 'E' above the data point, significant edge distribution on the test plot ($P < 0.01$). E below the data point, significant edge distribution on control plot ($P = 0.035$). —◆—, test plot; --□--, control plot.

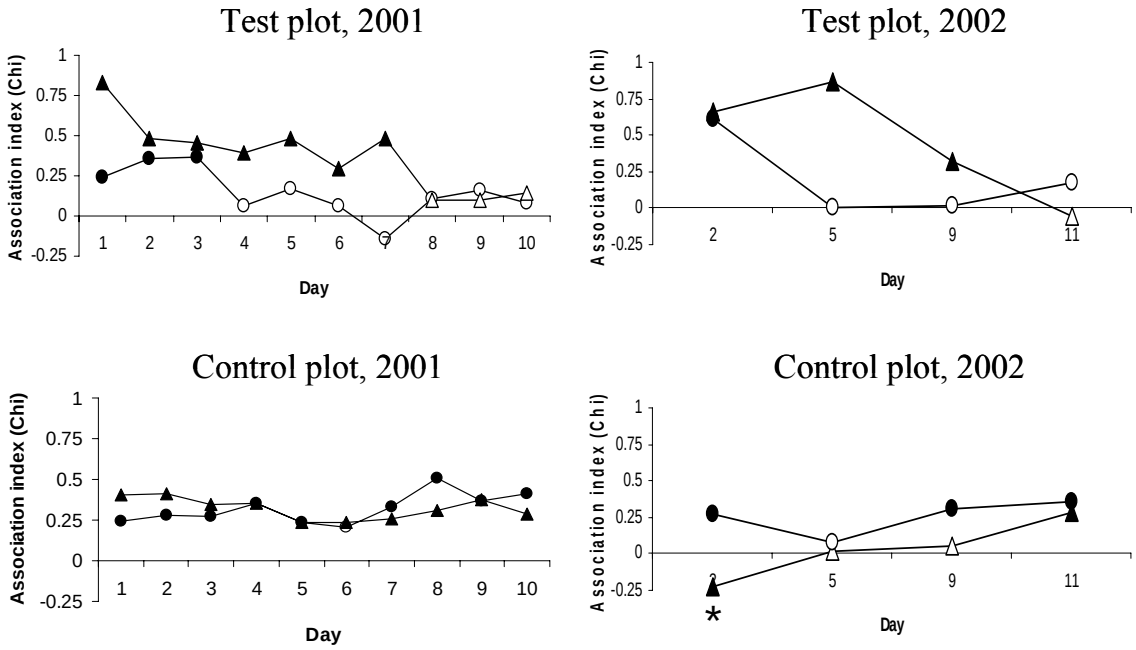

Figure 4. SADIE analysis of the spatial association between pollen beetles and plant growth stage variables on test and control plots in 2001 and 2002. —●—, significant spatial association between racemes in bud and pollen beetles; —O—, no significant spatial relationship between racemes in bud and pollen beetles; —▲—, significant spatial association between racemes in flower and pollen beetles; —△—, no significant spatial relationship between racemes in flower and pollen beetles. *, significant dissociation between racemes in flower and pollen beetles. All significance levels $P < 0.05$.

Relationships between distributions of plant growth variables and pollen beetles

Pollen beetle numbers were significantly ($P < 0.05$) spatially associated with one or both growth stage variables on 71 % of days for the test plot and 93% of days for the control plot (Fig. 4). In test plots in both years, the strength of the spatial associations of pollen beetles with both plant growth variables declined to insignificance over the course of the experiments (Fig. 4) and pollen beetle numbers were usually more strongly associated with racemes in flower than with racemes in bud (Fig.4).

Discussion

This study has improved our understanding of how the responses of pollen beetles to the dynamics of available food and oviposition sites on the OSR plant can influence pollen beetle spatial distributions within the crop. The results of this study have also shown how differential inflorescence growth stage might be used alone within a trap crop strategy to manipulate the spatial availability of these resources and thereby alter the spatial dynamics of the beetles on the crop. This confirms observations made by Hokkanen et al. (1986), but this study has additionally shown that buds should be abundant within the trap crop to prevent re-distribution onto the main crop. This highlights the importance of identifying cues/resources that determine pest residence on plants when developing trap crops and push-pull strategies, as a trap crop is only successful if the pest is retained there during the damage susceptible growth stages of the main crop (Hokkanen, 1991).

In both years, the experimental design achieved the desired aim of providing two contrasting plots with plant inflorescence growth stages manipulated to simulate a standard field crop (control) and a crop with a trap crop border (test). The control plots had neither edge nor centre distributed populations of racemes in flower or racemes in bud (Fig. 1). The test plot simulated 'trap crop strategy b', as described by Hokkanen (1991), in which the trap crop is the same species as the main crop; the trap crop is timed to be at its most attractive growth stage at the critical time for pest control when the main crop is most vulnerable, although it is less attractive. At the start of each experiment, outer plants (which constituted the trap crop) were at an attractive and resource rich growth stage for pollen beetles (i.e. at early-flowering stage), whilst the inner plants (like the control plots) were at the vulnerable late-bud stage. The duration of the experiments was sufficient to allow the inflorescence growth stage of the inner plants to change over the course of the experiment from the vulnerable late-bud stage through to the less vulnerable early-flowering stage, enabling the dynamics of pollen beetle distributions to be monitored in relation to changes in the distribution of resources at this critical time. In the test plot in both years, racemes in flower maintained a significant edge distribution throughout, but in contrast, racemes in bud changed from being edge distributed to being centre distributed (Fig. 1). This reflected the growth stage changes in the inner and outer plants. At the end of the experiments, outer plants of the test plot were at mid- to late-flowering stage with an increased ratio of racemes in flower to racemes in bud while the inner plants were at early-flowering stage with relatively more racemes in bud than the outer plants.

Inflorescence growth stage characteristics were shown to be important in determining the spatial distributions of pollen beetles, whose numbers were usually associated with one or both growth stage variables. These associations were weakest towards the end of the experiments on the test plot (Fig. 4), when plants on the edge were in mid- to late-flowering stage and probably less attractive. Pollen beetles on the test plot were usually more strongly associated with racemes in flower than racemes in bud, which concurs with other studies that show flowers provide more important cues than buds for pollen beetle host selection during inflorescence growth stages (Free and Williams, 1978; Cook et al., 2002a).

The single outer rows of earlier flowering plants on the test plots effectively simulated a trap crop scenario. Although, at first, the test plots attracted more pollen beetles than the control plots, they concentrated populations away from the inner plants (main crop) for at least a week; so much so that in 2002 there were consistently fewer beetles on the inner plants of the test plot than on inner plants of the control plot (Fig. 2). Significant edge distributions of pollen beetles were observed on the test plots each day for the first 7 and 10 days of experiments in 2001 and 2002, respectively, but only once on the control plot (day 10, 2002).

The edge distribution on the test plot was lost in the last few days of each experiment. At the same time as pollen beetles ceased to be edge distributed on the test plots, they also lost their spatial association with inflorescence growth stage variables. This loss did not occur on the control plots and probably occurred because counts of racemes on the older outer plants of the test plot were no longer a good measure of the abundance of floral resources they offered, older plants having fewer buds and flowers per raceme.

The loss of pollen beetle edge distribution coincided with a loss of edge distribution in racemes in bud, but not in racemes in flower. This implies that the loss of racemes in bud from the trap crop plants caused re-distribution of pollen beetles towards the inner plants. Buds and flowers provide food resources (Ekbom and Borg, 1996), but only buds provide oviposition sites. As the edge distribution of racemes in flower persisted throughout both experiments, it is likely that the loss of pollen beetle edge distribution was due to a reduction in available oviposition sites at the edge. The availability of buds is likely to be an important determinant of pollen beetle residence time on plants.

The effectiveness of the trap crop strategy is thus very dependent on the correct timing of the relative inflorescence growth stages of trap and main crop (Hokkanen et al., 1986). This study suggests that for optimum effectiveness in protecting a main crop at its vulnerable green bud stage, the trap crop border should then be at early flowering stage, when it has optimum attractiveness for pollen beetles and should continue to provide both buds and flowers for the damage susceptible period.

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Trap plants to avoid insecticide application against pollen beetles in oilseed rape

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Abstract. Rape seed was mixed with 2% turnip rape seed to produce trap plants within the oilseed rape crop. The turnip rape plants were up to 10 days faster in their development, which made them much more attractive to pollen beetles at early growth stages of the rape crop. At the most vulnerable growth stage of the rape plants, the turnip rape plants attracted 10 times more beetles. As the threshold for control is not usually exceeded by much, chemical control can be avoided in some years by sowing an admixture of rape seed with turnip rape.

Key words Trap crop, *Meligethes*, oilseed rape, turnip rape

Introduction

Insecticide treatment against pollen beetles is usually a mandatory measure in Swedish spring rape and also often in winter rape. Plants are most sensitive to economic damage in early bud stages, reflected in low control thresholds (Nilsson 1987). Pollen beetle females can live from pollen of any plant species, but the larvae can only develop in buds and flowers of cruciferous plants. Females will almost only oviposit in buds 2-3 mm long. If smaller buds are used they will often abort and larger buds will soon become flowers, forcing the young larvae to migrate to other flowers, and be exposed to predation and parasitism (Nilsson 1988). When pollen beetles arrive at an early growth stage, there are no buds big enough for egg laying and the females will feed on small buds. If there are bigger buds available these are preferred. Plants ahead of the others in development will be heavily attacked. If plants of turnip rape, that develop up to 2 weeks faster than rape plant, are present, pollen beetles will aggregate on these plants.

Hokkanen *et al.* (1986) surrounded cauliflower fields with a spring turnip rape crop which was regularly sprayed with insecticides. This made pollen beetle control almost unnecessary in the main crop. Büchi (1989, 1990, 1995) mixed the rape seed with 2% turnip rape seed but found that this was not enough to prevent damage to the rape crop. However, in these trials the pollen beetle population density was rather high, and the result could be different closer to the control threshold.

The objective of the experiments reported here was to see if a trap crop could divert the pollen beetles from the rape to lower the number of beetles per plant on the rape plants so that the control threshold was not exceeded. Provided that the seed quality of the turnip rape is the same as that of the rape crop it could be possible to mix rape seed with turnip rape seed, producing a trap crop integrated into the rape crop. The potential for use of turnip rape as a trap crop depends on the density of pollen beetles per plant in relation to the control threshold, which in turn is due to pollen beetle and turnip rape density per m^2. If the beetle density on the turnip rape plants is high, the female competition for suitable buds will force many beetles to move to other plants, other parts of the crop or other rape crops. Thus the thresholds for

control of the beetles on the rape plants can be exceeded. During 1991 to 1993 this balance was investigated in field trials in summer rape/summer turnip rape (Nilsson 1996) and verified in winter rape from 1995 to 1999.

Material and methods

Field trials, located in the southern part of Sweden, with 4 randomised blocks, were used. Two plots had only spring rape, two spring rape with 2% turnip rape mixed with the seed at sowing, and in the first year, there were also two plots with 4% turnip rape in each block. One of the two plots of each kind was sprayed with insecticide every 4-5 days to keep it free of pollen beetles.

Beetles were counted 2-3 times a week during bud stages. A plant in a row was chosen at random. Every 10^{th} plant from this starting point was identified, on which beetles were counted and plant growth stage determined. Five plants at two positions in each plot were sampled. The distance between the starting point and the 50^{th} plant were also measured, and together with row-width, this made it possible to calculate the number of plants per m^2. The number of turnip rape plants was also recorded. In plots with turnip rape plants, beetles were also counted and growth stage (GS) determined on 10 turnip rape plants using the same methods as described above. In total 12,000 plants were assessed in 9 field trials.

The results of the spring rape experiments were confirmed for winter rape in a 3 ha. demonstration field with a 2% mix of turnip rape and a 1 ha field without any trap crop. In these fields, beetles, plant growth stages and plant densities were recorded with the same methods as those used in the spring rape field trials. Winter rape plant densities varied from 30 to 100 plants/m^2. All crops were managed according to the farm practice in Sweden and common varieties were used.

In this study, the developmental stage scale of Harper & Berkenkamp (1975) was used. Stages in this key are here referred to as developmental stages (DS) to distinguish them from the growth stages of the BBCH key. This means that DS 2 is equivalent to GS 50-52 (early bud stage), DS 3 is GS 53-57 (middle bud stage), DS 4 is GS 59 (late bud stage) and DS 5 is GS 60 (flowering).

To be able to compare the developmental rates for the two plant species DS 3 was set to day 0 and developmental rate curves for individual fields arranged around this point to eliminate differences between years.

Plot size in spring rape was around 50 m^2, of which 25 was harvested with a plot combine at maturity. Only 7 of the 9 spring rape trials could be harvested. Yield samples were collected at harvest and analysed for water, oil and chlorophyll content. Pyrethroids at standard dose were applied using a field experimental sprayer. Plant samples were checked for differences between plots in pod midge and seed weevil damage about two weeks after the end of flowering.

Results and discussion

Developmental rate of plants

There is no difference in developmental rate between rape and turnip rape in spring crops. During bud stages turnip rape develops about 10 days earlier than spring rape (Fig. 1). In exceptional years and locations the difference is smaller. In winter crops, there are fewer observations, but they indicate that the difference in development of the two crops is smaller, around 5-10 days. Also the variation seems to be greater (Fig. 2).

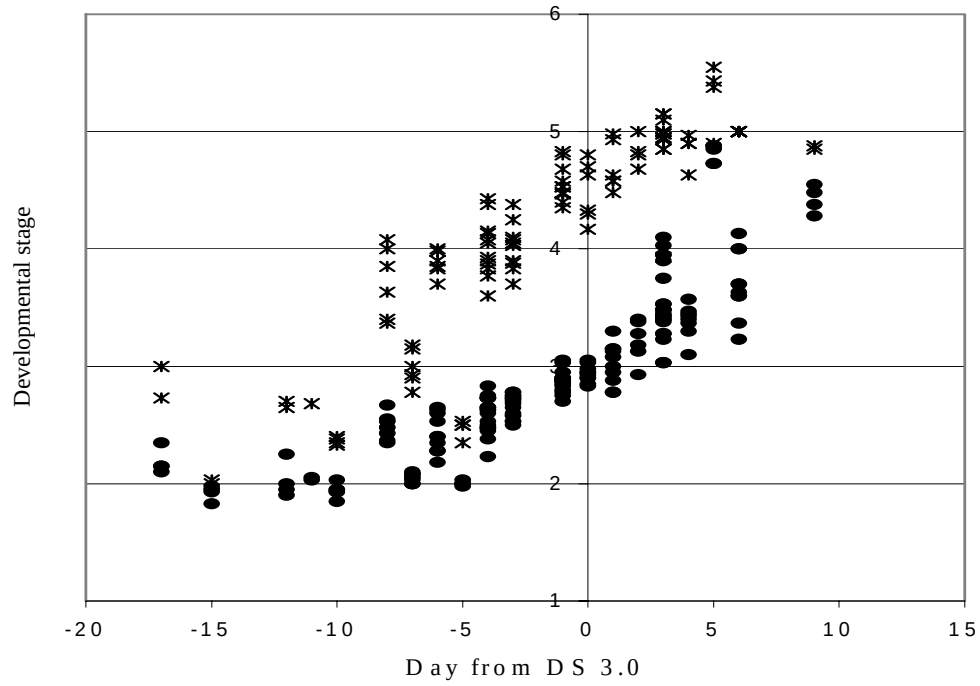

Figure 1. Developmental rate of spring rape (dots) and spring turnip rape (crosses). Trials 1991-1993.

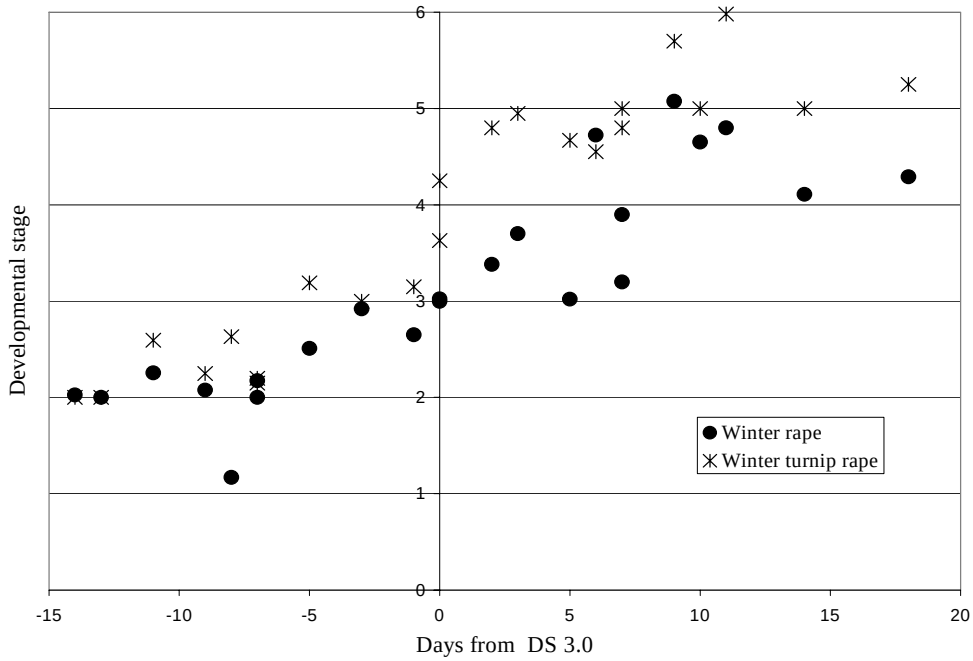

Figure 2. Developmental rate of winter rape and winter turnip rape. Observations 1995-98.

Distribution of beetles: plant development

Pollen beetles migrate to the oilseed crop to oviposit, and for this buds 2-3 mm in length are the most suitable (Nilsson 1988). Maximum occurrence of such buds is usually at early

flowering (GS 60; DS 5). As rape starts to form buds of suitable size for oviposition, there will be an increasing migration of females to the rape plants and rape GS 59 (DS 4) will usually be the end of the period when the turnip rape plants can give any protection to rape plants.

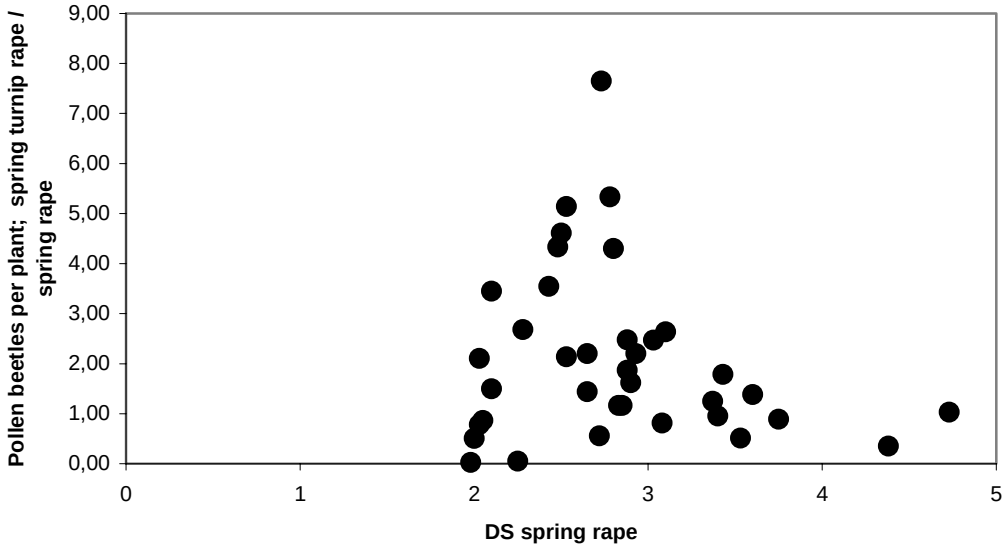

Figure 3. Quotient of pollen beetles on spring turnip rape and spring rape to developmental stages of spring rape

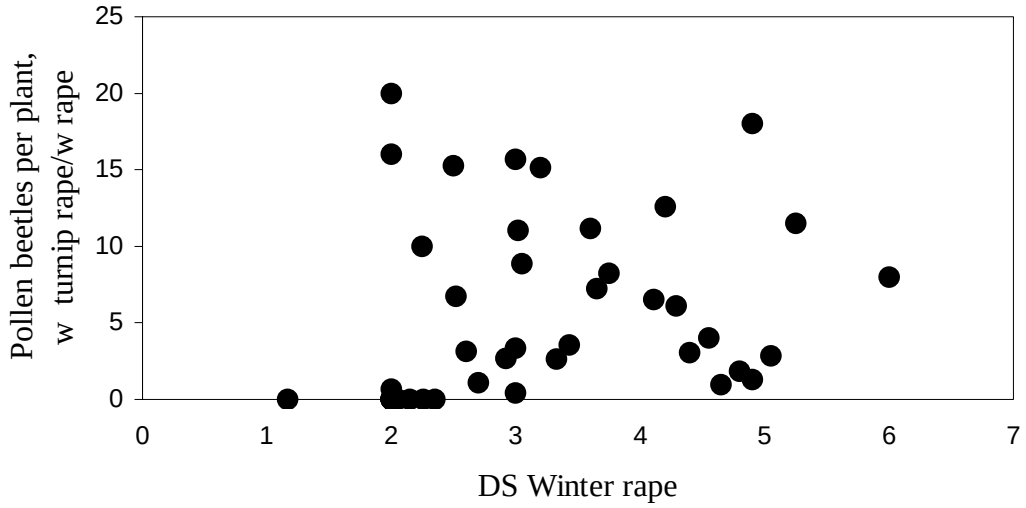

Figure 4. Quotient of pollen beetles on winter turnip rape and winter rape to growth stages for winter rape.

At most, about 20 times more pollen beetles have been recorded on the turnip rape plants compared to the rape plants. The quotient of pollen beetles per turnip rape plant to pollen beetles per rape plant reaches a maximum at GS 51/52 and approaches gradually one at late bud stages or first period of flowering (Figure 3 and 4).

The greater the difference in development between the two species, the greater the difference in pollen beetle density, illustrated for spring oilseed rape in figure 5. This starts when about 25% of the turnip rape plants have reached a higher growth stage than the rape plants.

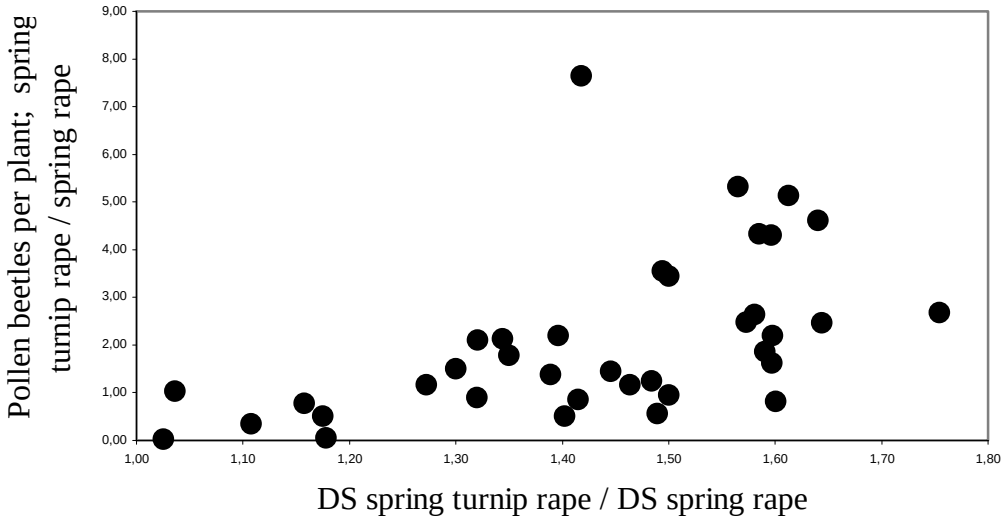

Figure 5. Quotient of pollen beetles on spring turnip rape and spring rape to quotient of growth stages for spring turnip rape and spring rape.

Distribution of beetles: turnip rape plant density

The effect of the turnip rape plants is also dependent on the number of turnip rape plants per m^2. The number of pollen beetles per plant for rape and turnip rape plants at different turnip rape plant densities is shown in Figure 6 and 7. The variation is, of course, substantial as data for different growth stage is used, but the effect increases up to about 10 plants per m^2, when almost all beetles will be found on the turnip rape plants.

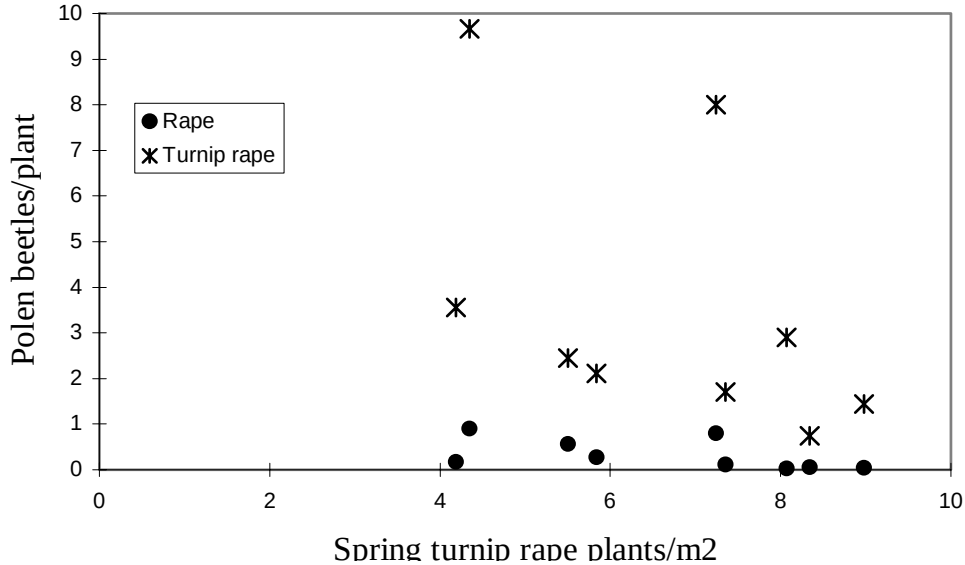

Figure 7. Pollen beetles per spring turnip rape and spring rape plant at different spring turnip rape plant densities

Practical application

The yields of the spring rape trials were low, between 0.5 and 0.7 tonnes of oil per hectare, drought being one of the reasons. The difference between untreated and insecticide treated plots is on the average 20% and highly significant (P<0.98). These yield losses comes from trials where pollen beetle densities on spring rape plants have exceeded the control threshold, in spite of the turnip rape plants.

In winter rape however, control thresholds were exceeded in the plot without turnip rape plants four times between 1994 and 2002, but in the plot with turnip rape only twice.

As always, there is a great variation between localities in plant densities. Rape plant densities are up to 25 plants / m^2 lower in the presence of turnip rape plants when plant densities in plots with only rape plants were between 150-250 plants/m^2, but this had no influence on yields.

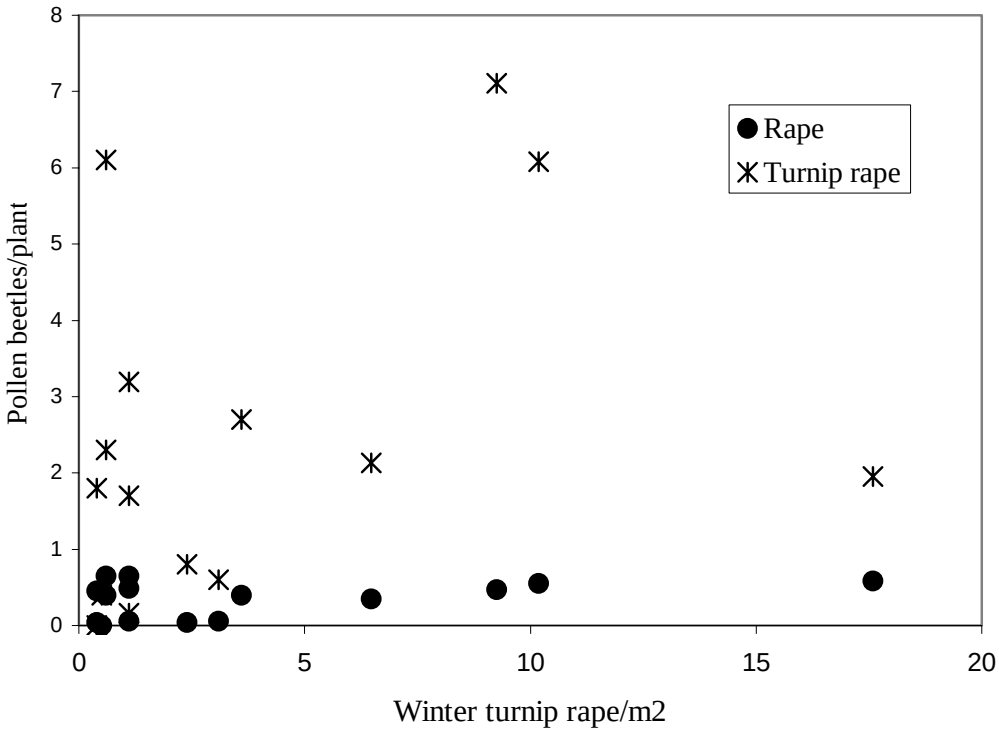

Figure 8. Pollen beetles on winter turnip rape and winter rape plants at different winter turnip rape plant densities.

Damage during the early growth stages of the rape plants will trigger more compensation responses and result in higher yield losses (Nilsson 1988). When buds begin to form on the rape plants, turnip rape plants have already got some buds suitable for egg laying and many more will soon form. The use of trap plants intermixed with the rape crop should evidently function. The limitations to this is the intraspecific competion among the females. When the population density is high (females per bud of the right size) the beetles will start searching for new plants. In a crop without species mix, this means that they start migrating away; in a crop with species mix they move to adjacent rape plants.

A disadvantage of this system is that it offers the pollen beetle a longer period for oviposition. The rate of plant development (i.e. the number of buds of optimal size) is one of the limiting factors for population increase in pollen beetles (Nilsson 1988). This bud

population is now increased as two species of plant with temporal differences in development are available to the pollen beetle females.

Moreover it could be argued that a crop with turnip rape mixed with rape would attract more pollen beetles than if there had been no turnip rape in the field. Neither the field trials reported here nor a small study in farmers' fields however indicates this.

In spring rape, 2% turnip rape plants is around 4-5 plants per m^2., but in winter rape, where plant density is 50 plants per m^2 or less, this is only 1 plant per m^2. The control thresholds in spring rape is 3-6 times higher than in winter rape, at least in Sweden, which makes the performance of the two systems more comparable. At pollen beetle densities close to the control threshold, turnip rape plants should increase the control threshold at least 10% and consequently have an effect on the number of sprays needed to protect the crop from economic damage. The effect will be of practical importance in winter rape in the first place. If practised by many farms, insecticide use will decrease which should give parasitoids and other natural enemies more importance and thereby increase the effect of the trap crop. Moreover the effects of an insecticide spray will also be enhanced by the trap crop which will collect beetles arriving at the crop and thereby increase the duration of the treatment effect.

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Effect of sowing density of oilseed rape on the abundance and within-plant distribution of cabbage stem flea beetle, *Psylliodes chrysocephala*

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Abstract: In randomized field plot experiments in Germany the effects of plant density and plant architecture on infestation of oilseed rape by cabbage stem flea beetle, *Psylliodes chrysocephala* L., were investigated in the autumn of 1999, 2000 and 2001. Various sowing rates (30, 60 and 90 seeds/m²) were applied to produce different plant densities. The lowest sowing rate resulted in larger oilseed rape plants with significantly increased numbers of leaves and lateral buds. The number of *P. chrysocephala* larvae/m² declined with decreasing numbers of plants/m². In contrast, the number of larvae/plant significantly increased at low plant densities. The within-plant distribution of the larvae was only affected in the year 2000 when the vigorous growth of the hybrid cultivar in the autumn allowed an early development of lateral buds, particularly at the lowest plant density. Under these conditions, at higher sowing rates (60 seeds/m², 90 seeds/m²) the number of larvae within the terminal buds was significantly higher than at the low sowing rate (30 seeds/m²). Larger petioles and lateral buds of low density plants obviously provided a sufficient food resource for the larvae, thereby preventing the migration of larvae to the terminal bud. These results indicate that the risk of most harmful infestations of the terminal bud which often causes a high overwinter mortality of plants can be reduced by growing oilseed rape at low plant densities.

Key words: *Psylliodes chrysocephala,* oilseed rape, plant density, within-plant distribution.

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Cultivation techniques as means to control pests in organic oilseed rape production

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Abstract: A field trial was conducted to compare the effectiveness of different husbandry practices in controlling pests in organic winter oilseed rape production. In oilseed rape, considerable damage and harvest losses can be caused by the cabbage stem flea beetle (*Psylliodes chrysocephala*), the pollen beetle (*Meligethes* spp.), the cabbage stem weevil (*Ceutorhynchus pallidactylus*), the oilseed rape stem weevil (*C. napi*), the cabbage seed weevil (*C. obstrictus* syn. *assimilis*) and the Brassica pod midge (*Dasineura brassicae*). A farming system that integrates means to promote natural enemies (mulching, a seed rate of 3.5 kg/ha, field margin weed strips, trap crop strips, drilling in a wide row (50 cm) and use of a hoeing machine for weed control in autumn and spring = **mulching/hoeing** treatment) was compared to a standard system (ploughing, conventional drilling technique, comb harrowing in autumn and spring, a seed rate of 5.0 kg/ha, no weed-strips or trap crop strips = **ploughing/comb harrowing** treatment). A third system, a **mulching/comb harrowing** treatment (mulching, a seed rate of 5.0 kg/ha, with weed- and trap crop strips) was also compared. The results were as follows:

- An acceptable yield was achieved. In the ploughed treatment the yield was 2.65 t/ha; 20% higher than in the mulching treatments.
- Trap crop strips (cv. Express) which were sown in the treatments with **mulching** showed (compared to field edges without trap crop strips) a higher infestation by the Brassica pod midge, the cabbage seed weevil and the pollen beetle than the crop plants within the field (cv. Oase). For the cabbage stem weevil however no significant differences were recorded.
- While the Brassica pod midge achieved the highest reproduction rate within the **mulching/hoeing** treatment, for all other pests the highest reproduction rate was recorded in the **ploughing/comb harrowing** treatment.
- The highest abundance of predators active on the soil surface was recorded in the **mulching/comb harrowing** treatment. Due to the higher abundance of natural enemies, the reproduction rate of all pest species (except the pollen beetle) was lowest in the **mulching/comb harrowing** treatment. Thus, in this treatment considerably more larvae had to be invested to produce one mature individual of the new pest generation.

All treatments showed advantages and disadvantages: the **ploughing/comb harrowing** treatment achieved the highest yield, but also the highest hatching rates of pests of the new generation (except the Brassica pod midge). The **mulching/comb harrowing** treatment promoted natural enemies, but there is a higher risk of yield losses due to weeds. In the **mulching/hoeing** treatment, the reduction of the weed risk by mechanical means was followed by a reduction in numbers of natural enemies. Furthermore, in this treatment a higher infestation by the Brassicae pod midge and other occasional pests occuring in autumn (e.g. *Athalia rosae, Plutella xylostella, Myzus persicae*) was recorded.

This one-year field trial clearly demonstrated that organic oilseed rape cultivation is potentially possible if relevant husbandry practices are considered and used in an appropriate way.

Key words: Oilseed rape pests, organic, tillage, mulching, row width, trap crop, *Meligethes* spp., *Ceutorhynchus* spp., *Dasineura brassicae*, *Psylliodes chrysocephala*

Introduction

The cultivation of organic winter oilseed rape in Germany is still of negligible importance. For instance, the agronomic report of the German Ministry of Consumer Protection, Food and Agriculture shows no input for organic oilseed rape production (Bundesministerium für Ernährung und Landwirtschaft 1998). Therefore, most experience originates from experimental oilseed rape growing (e.g. Haas & Kramer 1995, Loges & Böhm 2001, Mayer et al. 2001, Paulsen et al. 2003) or from other countries, mainly Scandinavia (Hokkanen & Piirainen 1995, Petterson et al. 2002), which however deal mainly with spring rape. Nevertheless, organic oilseed rape is expected to become increasingly important for several reasons:

1) Rapeseed cake is excellent as protein fodder. If in the future, fodder for cows and pigs should originate exclusively from organic production, rapeseed cake could become extremely important (FOER 2003).
2) Organic vegetable oils are usually made from sunflower, soya or olive oil; they are produced mainly outside the EU and soya oils are often produced at the cost of clearing (sub) tropical rainforests (Dreyer, Öko-Ring Lower Saxony, pers. comm.. 2003).
3) Should organic farms decide to work completely on an organic basis, they should be able to grow their own fuel to cover their own consumption. Oil from rape seed would, in this situation, be the first choice considering the climatic conditions in central Europe (Paulsen 2003, von Bonin 2003).

There are some approaches to control oilseed rape pests by means which potentially could be suitable for organic oilseed rape production. In Finland, field experiments to investigate control of pollen beetles by pathogens e.g. protozoans like *Nosema meligethi* were implemented (Hokkanen et al. 1988, Hokkanen 1989). Recent approaches integrate in addition entomopathogenic fungi like *Metarhizium anisopliae, Beauveria bassiana* and nematodes such as *Steinernema feltiae* and *St. carpocapsae* to control *Phyllotreta* spp. and *Meligethes* spp. (Hokkanen and Menzler-Hokkanen 2003). Field trials with *St. feltiae* were particularly promising, resulting in a reduction of pollen beetles by 93.8%. However, unfortunately the key parasitoid of pollen beetles *Phradis morionellus* was also reduced by 94.4% (Hokkanen 2004). In Sweden in organic oilseed rape production, pollen beetles were successfully controlled by means of vibrating trays (Nilsson, pers. comm.) which were used until the 1960s even in conventionally grown oilseed rape fields (Büchs 1998). Improved apparatus were recently tested in organic oilseed rape (Makowski, 1998). However, the techniques mentioned above need special investments (e.g. vibrating trays) and/or have to be further developed technically to be accepted by farmers for practical application (e.g. pathogens). Therefore, the goal of the research presented in this paper was not to test active measures for pest control in organic oilseed rape production, but to compare the effects of different crop management procedures (husbandry practices) which are already more or less established and thus, currently available for organic farmers, on the occurrence and control of rape pests. Results show that certain measures e.g. mulching (House & Stinner 1983, Haskins & Shaddy 1986; Heimbach et al. 1997, Ulber 2003), trap crops (Büchi 1995, Hokkanen 1991, Cook et al, 2002), strips of wild flowers (Hausamman 1996, Lethmayer et al. 1997, Thies et al. 1997, 2000; Thies & Tscharntke 1999), row width, seed rates (Ulber and Wedemeyer 2004, this volume) are suitable for mobilising the natural regulation potential of pests. For example, polyphagous predators are encouraged by careful soil tillage and organic biomass accumulation (e.g. mulching) (Büchs 2003). These and other measures should be verified under real organic crop-growing conditions in the field experiments presented here.

Materials and methods

In accordance with organic farming guidelines (e.g. European Commission 1991, 2002), a plough/comb harrowing treatment was compared to two mulching methods.

***Plough/comb harrowing treatment*:** conventional soil tillage by ploughing, drilling technique according to current practice with a seed rate of 5.0 kg/ha, 10 cm row width, cultivar Oase, no trap crop, and no special field margin vegetation. Weed control by comb harrowing in autumn (11 September) and spring (25 March).

***Mulching/comb harrowing treatment*:** soil tillage without ploughing (mulching), drilling technique according to current practice with a seed rate of 5.0 kg/ha, 10 cm row width, cultivar Oase, a 3.0 m-wide trap crop with the early-flowering rape cultivar Express, a 2 m-wide wild flower strip comprising many umbellifers, weed control by comb harrowing in autumn (11 September) and spring (25 March).

***Mulching/hoeing treatment*:** soil tillage without ploughing (mulching), drilling in a row space of 50 cm with a seed rate of 3.5 kg/ha, cultivar Oase, a 3 m-wide trap crop and 2 m-wide wild flower strip as above; weed control by hoeing in autumn (11 September) and spring (25 March).

***Management*:**

Previous crop: yellow peas (harvested 9 August 2002)

21.08.2002:	Ploughing (ploughing/comb harrowing treatment) Wing share cultivator (mulching treatments) Implementation of two 2 m-wide wild flower strips by drilling of a special seed mix (mulching treatments) Implementation of two 3 m-wide winter oilseed rape trap crop strips by drilling of cv. Express" (mulching treatments) Drilling of the remaining field area with cv. Oase in the mulching as well as in the ploughing treatments
03.09.2002	Installation of 4 yellow water traps and 4 pitfall traps per treatment, managed weekly until 5 November 2002 and from 11 March 2003 until end of flowering, managed every 14 days
11.09.2002	Hoeing (mulching/hoeing treatment) and comb harrowing (other two treatments)
20.11.2002	Sampling of 60 plants/treatment to assess infestation of *Psylliodes chrysocephala* (larvae) and assessment of adult feeding marks following EPPO-guidline PP 1/73 (3) (EPPO 2002). Crop plant density assessment
23.03.2003	BBCH growth stage 55 (Lancashire et al, 1991): Sampling of 50 plants/treatment to assess spring infestation by *Psylliodes chrysocephala* (larvae) in spring
25.03.2003	Hoeing (mulching/hoeing treatment) and comb harrowing (other two treatments); Crop plant density assessment
16.04.2003	Beating of at least 40 plants/treatment (and 20 plants/trap crop or field margin) to assess infestation by *Meligethes* spp, *Ceutorhynchus pallidactylus* and *C. assimilis* every 4-5 days until 28.05.2003 Weekly assessment of buds (main raceme and third secondary raceme) for infestation and feeding damage by *Meligethes* spp. and *Ceutorhynchus pallidactylus* until 13.05.2003
30.04.2003	Installation of 9 funnel traps per treatment, maintained weekly until 10.07.2003

20.05.2003 Sampling of 100 plants/treatment and 30 plants/trap crop (mulching treatments) or field margins (ploughed treatment) to assess the infestation by larvae of stem weevil (*Ceutorhynchus pallidactylus, C. napi*)

02.06.2003 Sampling of 30 plants/treatment and 20 plants/trap crop (mulching treatments) or field margins (ploughed treatment) to assess pod numbers and their infestation by larvae of *Dasineura brassicae* and *C. assimilis*

05.06.2003 Installation of 9 emergence traps/treatment and 1 emergence trap/trap crop (mulching treatments) or field margins (ploughed treatment)

14.07.2003 Harvest. All traps removed and re-installed after harvest was finished

The experimental field (1.4 ha) was located in the south-east of Lower Saxony, approx. 10 km south of Braunschweig near Ahlum, Germany. The field has been managed organically since 1995 with an 8-course crop rotation including summer wheat, summer wheat with catch crop clover grass, pig beans, winter barley, summer wheat, winter rye, yellow peas and oilseed rape. The experimental field was surrounded by other fields as there was a fallow with Phacelia/Alexandrinian clover (organic; west) and fields with vegetables (leek) (organic; east), winter barley (conventional; south), potatoes (conventional; north) and two grassy field tracks (south and north).

The oilseed rape was harvested on 14 July 2003 using a plot harvest combine. Six strips of 2 m width and 20 m length from the central area of each treatment were harvested to determine the average yield.

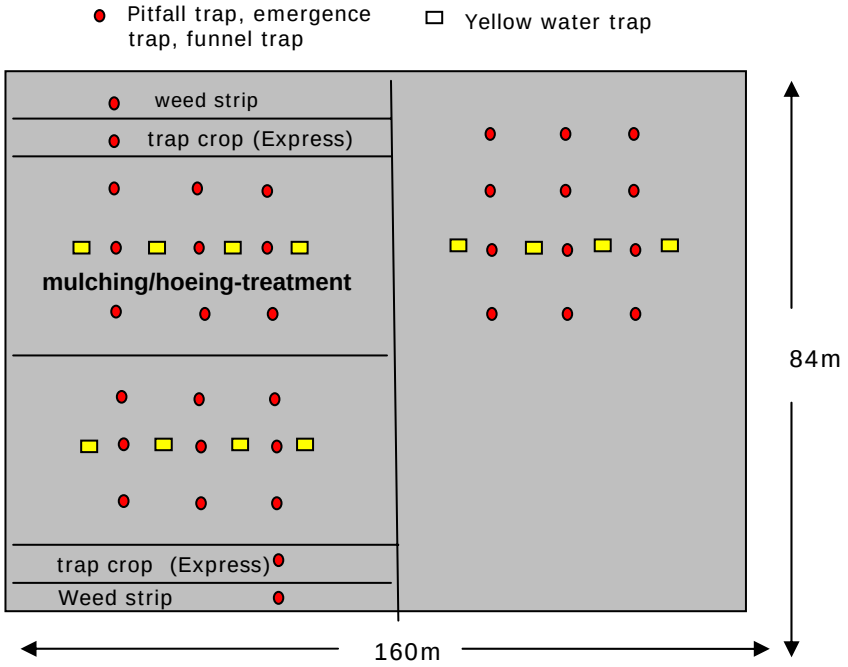

Fig. 1: Design of the experimental field for organic winter oilseed rape production

Results and discussion

Yield

Despite the extreme drought in Germany in the summer of 2003, the winter oilseed rape showed an acceptable yield in all three systems, as far as organic farming is concerned: 2.12 t/ha (mulching/hoeing treatment), 2.10 t/ha (mulching/comb harrowing) and 2.65 t/ha

(plough/comb harrowing treatment). The yield from the mulching treatments was thus around 20% less than in the ploughed treatment. However, in other cases of organic oilseed rape production, the yield is only about 1 t/ha (Paulsen et al. 2003).

Crop development

As a result of the very dry and warm conditions in September 2002, more oilseed rape plants emerged in both mulching treatments than in the ploughed system. Juvenile growth in autumn was very rapid. This led to high plant loss over winter following considerable frost periods without snow cover. Consequently, over winter plant density decreased drastically, particularly in the mulching/comb harrowing treatment, and spring density was nearly equal in all three treatments (approx. 40 plants/m²; Table 1).

Table 1. Seed rate and establishment of oilseed rape seedlings in autumn and spring

	mulching/hoeing	**muching/comb harrowing**	**plough/comb harrowing**
Seed rate	70 seeds/ m²	100 seeds/ m²	100 seeds/ m²
Seedling density autumn (20/11/02)	56 seedlings/ m²	79 seedlings/ m²	53 seedlings/ m²
Plant density Spring (25/3/03/03)	40 seedlings/ m²	40 seedlings/ m²	38 seedlings/ m²

Weed density

Weed density in autumn was highest in the mulching/hoeing treatment (because of the high area of open soil) with 260 plants/m². There were 210 plants/m² in the mulching/comb harrowing treatment and least in the ploughed treatment (171 plants/m²), in which the most plant species were also recorded (4.0 plant species/0.1 m² compared with 2.3 species in mulching/hoeing and 2.2 in mulching/comb harrowing treatments (Haeusler & Zwerger, unpublished).

Table. 2. Mean number of pests caught in yellow water traps during autumn 2002 accumulated between 03 Sept – 05 Nov (top 4 rows) and assessment of plant damage due to *Psylliodes chrysocephala* using feeding marks following EPPO-guidline PP 1/73 (3) (EPPO 2002) (bottom 2 rows). Highest values in bold.

Pest/damage	**mulching/hoeing**	**mulching/comb harrowing**	**plough/comb harrowing**
Psylliodes chrysocephala	0.3	0.5	**0.7**
Athalia rosae	**18.0**	13.3	12.9
Plutella xylostella	**11.0**	8.8	7.5
Myzus persicae	**20.4**	18.2	18.1
feeding damage 24.09.02	**4.1%**	3.9%	2.8%
feeding damage 20.11.02	0.7%	**0.9%**	0.5%

Pest immigration

Pest immigration in autumn was monitored by yellow water traps. Yellow water trap samples in autumn 2002 recorded an unusually intensive flight activity of the turnip sawfly (*Athalia*

rosae) and the diamond-back moth (*Plutella xylostella*), but extremely low numbers of immigrating *Psylliodes chrysocephala* were caught (Table 2). In Autumn 2003 (03 Sept - 02 Oct), the highest numbers of all pests, except for the rape stem flea beetle, were recorded in the water traps in the mulching/hoeing treatment and the lowest in the plough/comb harrowing treatment. The same trend was observed for the percentage of feeding marks (cut-out-hole-feeding) of *Psylliodes chrysocephala* on the leaves (Table 2). As the traps were dug in the soil so that the upper rim was at an equal level as the soil surface and hoeing had recently taken place (11 September; one week after the start of yellow water trap sampling) pest insects obviously preferred the very characteristic spatial pattern of rape plants and open soil in the mulching/hoeing treatment with the wide row space and oilseed rape plants in rows.

Trap crop

The 3m-wide trap crop was drilled at the margin of the mulching treatments with the cultivar Express. For a trap crop to be effective, it should be early-flowering with respect to the main crop (Hokkanen 1991; Cook et al, 2002). However, in the spring of 2002, there was a hot period at the time of the bud stage, which continued throughout the whole summer. Both oilseed rape cultivars progressed to full flower very rapidly. Consequently, the difference in the time of flowering of the 'early-flowering' trap crop and the entire field area was hardly distinguishable (only two days at most). However, cv. Express was not only chosen for the trap crop because it flowers much earlier compared to the crop cultivar Oase, but also because it had been proven to be particularly attractive to most oilseed rape pests, including slugs, in our 4-year-studies comparing different cultivars (Büchs, unpublished). Therefore, a "trap crop" can have an impact on pests, which has nothing to do with its time of flowering (see also Walters et al. 2003).

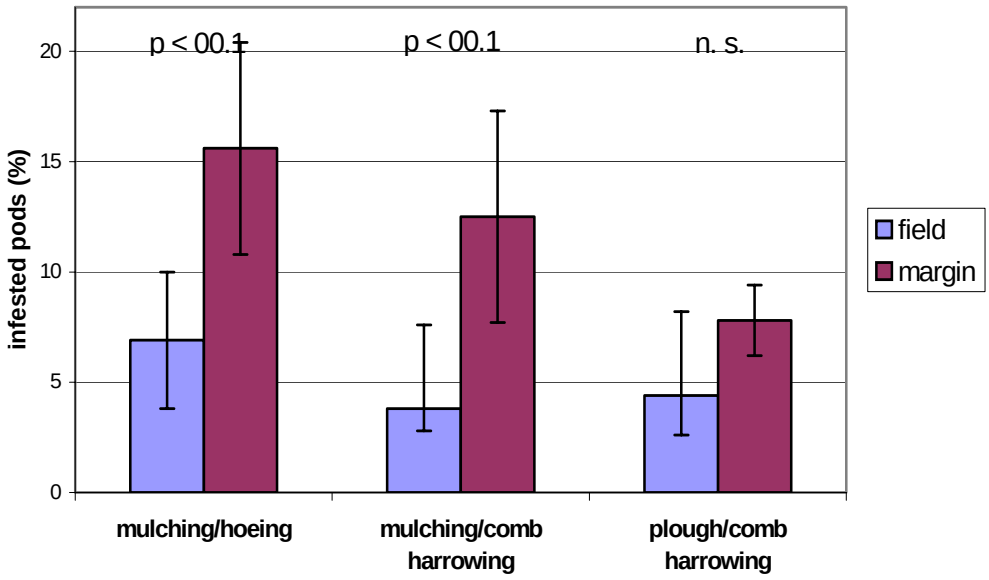

Fig. 2: Pod infestation (%) by Brassica pod midge (*Dasineura brassicae*) in central areas of the treatment (light columns) and in field margins with trap crop cv. Express (mulching/hoeing, mulching/comb harrowing) or without trap crop (plough/comb harrowing) (dark columns). Plant samples taken at BBCH stage 75.

In order to estimate the effects of the trap crop, pest infestation of the trap crop was compared: (i) with the infestation in the centre of the entire crop area of each treatment and (ii) with the infestation in the marginal area of the plough/comb harrowing treatment. The trap crop strips of the two mulching treatments had a significant effect on infestation by the Brassica pod midge (*D. brassicae*): the proportion of infested pods ranged between 12% and 16% in the trap crop strips, but in the field margin without trap crop only 8% were infested. The infestation of marginal area and central field area differed significantly ($p < 0.01$; Mann-Whitney-U-test) in the mulching treatments with the trap crop, whilst no significant differences could be recorded in the plough/comb harrowing treatment where no trap crop was grown in the margin (Figure 2).

Damage by insect feeding on the buds, e.g. by the pollen beetle (*Meligethes* spp.) or by the stem weevils (mainly *C. pallidactylus*) during maturity feeding are relevant to yield since the ovary of the plant can be destroyed, and secondary pests such as thrips (Thysanoptera) are able to enter the buds and damage them, reducing pod set. Fig. 3 shows that in the centre of each treatment the feeding damage to buds hardly differs (between 0.4 and 0.67%). Similarly, there was little difference in bud damage in plants from the field centre or margin in the ploughed/comb harrowing treatment. However the damage in the cv. Express trap crop of the mulching treatments was up to 4.75 times higher than in the field centre.

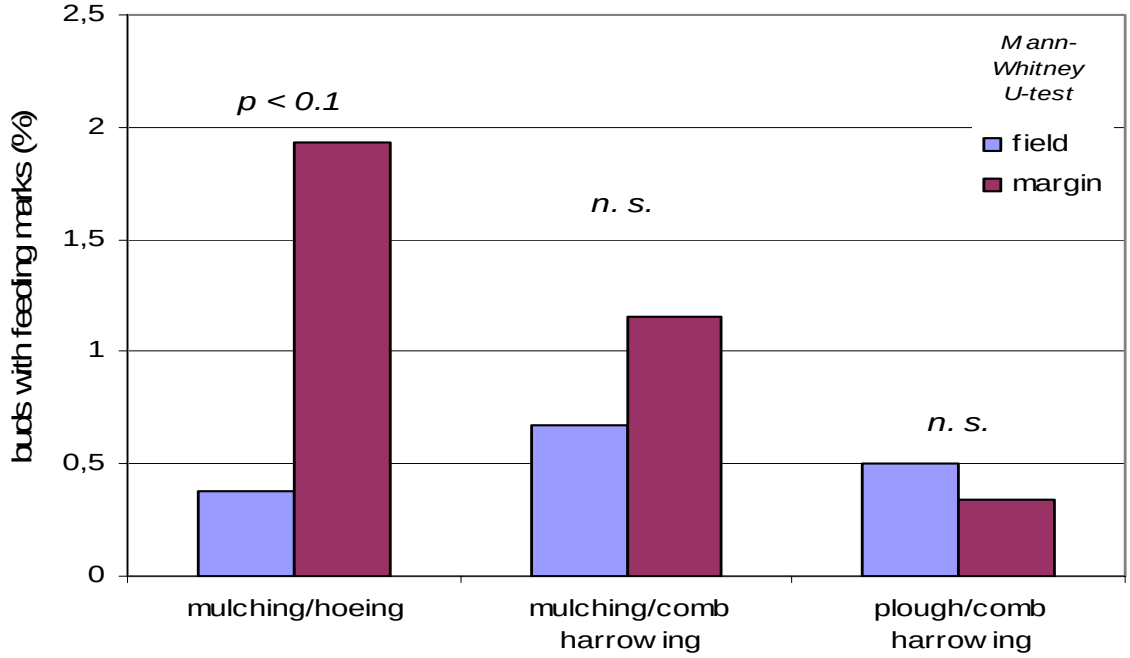

Fig. 3. Percentage of buds damaged by feeding of pollen beetles and stem weevils in central areas of the treatments (light columns) and in field margins with trap crop cv. Express (mulching/hoeing, mulching/comb harrowing) or without trap crop (plough/comb harrowing) (dark columns). Plant samples taken at BBCH-stage 55.

The stem weevil species *Ceutorhynchus pallidactylus* (cabbage stem weevil) and *C. napi* (rape stem weevil) immigrate into the crops in spring, which was assessed using yellow water traps. Eggs are laid into the plant shoots (*C. pallidactylus*) or stems (*C. napi*) and the larvae of both species mine the stems. Of the stem weevils caught in the yellow water traps, 94% were *C. pallidactylus* and 6% were *C. napi*. Plant infestation by the weevils was considerable,

amounting up to 83% with 1.1 - 2.4 larvae/plant on average in the field centres. In all three treatments the infestation of the field margin was clearly higher than in the field centre (Tab. 3; >90% infested plants with 3.3 - 4.2 larvae/plant). However, the infestation levels of the field marginal areas with and without trap crop were not significantly different, so no clear effect of the trap crop on the stem weevils was evident.

Table. 3: Oilseed rape infestation by stem weevil larvae (*C. pallidactylus* and *C. napi*): percentage of infested plants and no. of larvae/plant in central areas of the treatments and in field margins with or without a trap crop. Plant samples (100 plants/treatment field centre, 30 plants/field margin) taken at BBCH-stage 67-69.

Treatment	**Mulching/hoeing**		**Mulching/comb harrowing**		**Plough/comb harrowing**	
Location (field centre /margin) and cultivar	**centre** (cv. Oasis)	**margin** (trap crop cv.Express)	**centre** (cv. Oasis)	**margin** (trap crop cv. Express)	**centre** (cv.Oasis)	**margin** (cv. Oasis)
% infested plants	**83.0** (± 23.6)	**90.0** (± 11.0)	**77.0** (± 26.2)	**87.0** (± 16.3)	**81.0** (± 22.9)	**97.0** (± 8.2)
No. larvae /plant	**2.38** (±1.31)	**3.72** (± 0.79)	**2.2** (±1.71)	**2.67** (± 1.41)	**1.1** (±1.95)	**3.52** (± 1.29)

Table 4. Accumulated hatching rates (5 June - 5 August 2003) of the new generation of oilseed rape pest insects (mean numbers/m²) recorded from emergence traps placed in different systems of organic oilseed rape production. Highest values in bold.

Treatment	**mulching/hoeing**	**mulching/comb harrowing**	**Plough/comb harrowing**
Brassica pod midge (*Dasineura brassicae*)	**867** (±46.0)	624 (± 104.1)	543 (± 69.6)
Pollen beetle (*Meligethes* spp.)	10.5 (± 1.3)	12.6 (± 1.2)	**23.2** (± 3.1)
Cabbage seed weevil (*Ceutorhynchus obstrictus*)	86.3 (± 6.5)	51.6 (±7.5)	**155.8** (± 24.3)
Cabbage stem weevil (*C. pallidactylus*)	24.7 (± 4.2)	13.7 (± 1.0)	**37.9** (± 7.4)
Oilseed rape stem flea beetle (*Psylliodes chrysocephala*)	3.2 (± 0.5)	**6.3** (±1.7)	**6.3** (± 3.0)

Pest larvae which drop out of flower-heads, pods or rape stems to the ground to pupate in the soil were caught with funnel traps and the emergence of the new generation (adults) was recorded with emergence traps. With the exception of *Dasineura brassicae*, most of the individuals of the new pest generation (pollen beetle, cabbage seed weevil, cabbage stem weevil, cabbage stem flea beetle) emerged in the plough/comb harrowing treatment (Table 4). This suggests that the plough/comb harrowing treatment has the potential to generate the most pests, a fact which is of importance for the re-infestation of the following year's rape fields.

From the ratio of dropping larvae to emergence of the new pest generation the reproduction efficiency of the pests and thus, the efficiency of natural enemies in pest regulation can be estimated. Table 5 demonstrates that all pests, except for the pollen beetle, in the mulch/comb harrowing treatment require more larvae to generate one adult specimen of the new generation. This means that reproduction efficiency is lowest for pests when mulching/comb harrowing is applied. This indicates a higher density and efficiency of natural enemies (predators, parasitoids, pathogens) in the mulching/comb harrowing treatment compared to the other two treatments and is clearly demonstrated for the Hybotidae (dancing flies) and spiders which are apparently negatively affected by measures like hoeing and ploughing (Table 6).

Table 5. Reproduction rates of different oilseed rape pest insects (No. of larvae needed to produce one adult specimen of the new pest generation) calculated from the ratio of larval drop: hatching rate (per m²) (highest values in bold). Assessment of larval drop by funnel traps (30 April - 10 July) and of hatching rate by emergence traps (05 June - 05 August).

Treatment	**mulching/hoeing**	**mulching/comb harrowing**	**Plough/comb harrowing**
Brassica pod midge (*Dasineura brassicae*)	7.8	**20.7**	15.9
Pollen beetle (*Meligethes* spp.)	**5.6**	3.3	2.5
Cabbage seed weevil (*Ceutorhynchus obstrictus*)	0.29	**0.94**	0.48
Cabbage stem weevil (*C. pallidactylus*)	3.9	**6.4**	2.0

Table. 6. Accumulated numbers of predators in three systems of organic oilseed rape production. Spiders and rove beetles sampled with pitfall traps between 15 May and 26 June 2003, dancing flies sampled with yellow water traps between 03 Sept – 08 Oct 2002. Significant differences (Mann-Whitney-U-test) are marked by different characters. Highest values in bold.

	mulching/hoeing	**mulching/ comb harrowing**	**Plough/comb harrowing**	**P**
Spiders (Araneae)	685 ^{a}	**954** ^{b}	895 ^{ab}	≤ 0.05
Wolf spiders (Lycosidae)	36	**60**	17	n. s.
Rove beetles (Staphylinidae)	144 ^{a}	**198** ^{ab}	288 ^{b}	< 0.01
Ground beetles (Carabidae)	**624**	614	536	n. s.
Dancing flies (Hybotidae)	19.9^{a} (± 8.8)	**42.9^{b} (+- 15.8)**	10.8^{a} (± 2.2)	< 0.05

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Parasitoids

Integrated Protection in Oilseed Crops
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Occurrence of pollen beetle parasitoids in the south of Sweden

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Abstract: The three most abundant Ichneumonid parasitoid species attacking pollen beetles in southern Sweden are *Phradis interstitialis*, *P. morionellus* and *Tersilochus heterocerus*. The relative abundance of females of these species has been evaluated throughout two seasons in an area with both winter and spring rape fields. In the beginning of the season *P. interstitialis* was the predominant species, while later, during flowering of the spring rape, *P. morionellus* was the most abundant species. *T. heterocerus* was found in smaller numbers throughout the first season, but were more abundant during the second season.

Key words: pollen beetles, *Meligethes*, *Phradis*, *Tersilochus*, parasitoids, biological control

Introduction

Pollen beetles (*Meligethes spp*.) cause damage of economic importance to oilseed rape, especially in northern Europe (Alford et al, 2003). Parasitoids can be important in control of these beetles. Levels of parasitation by hymenopteran parasitoids above 50% have been observed in several studies (Winfield, 1963; Nilsson and Andreasson, 1987; Billqvist and Ekbom 2001b; Büchi 2002). Nine species of hymenopteran parasitoids attacking pollen beetle larvae are found in Europe (Nilsson, 2003).

The distribution of the different species depends on factors such as the type of crops grown in the vicinity and climate. In central Europe and the UK, the most abundant species, occurring on winter oilseed crops are one or more of the univoltine ichneumonids *Phradis interstitialis*, *P. morionellus* or *Tersilochus heterocerus* (Winfield, 1963; Klingenberg and Ulber, 1994; Büchi, 2002; Ferguson, et al. 2003). In Finland, and in areas of Sweden where mainly spring rape is grown, *P. morionellus* and the multivoltine *Diospilus capito* (Braconidae) are normally the most abundant species (Husberg and Hokkanen, 2001; Billqvist and Ekbom 2001a; 2001b). The relative abundance of the ichneumonid parastoids has been evaluated in an area of southern Sweden throughout two seasons.

Materials and methods

Samples of parasitoids were taken from crops grown on the university farm at Alnarp near Malmö in the south of Sweden. In both years fields with winter rape and fields with spring rape were located within 300 m from each other. The winter rape fields were 3 ha, while the spring rape fields were smaller (approx. 0.4 ha). The fields were not sprayed with insecticides during the sampling period. Insects were caught with sweep nets within 10 m from the edges of the fields from the uppermost part of the oilseed rape canopy as well as from above it. All female hymenopteran parasitoids of the superfamily Ichneumonoidea and that were of the approximate size of pollen beetle parasitoids were collected from the net with an aspirator.

Insects were collected between 9 am and 2 pm on days without rainfall and low wind. At the time of the season when flowering of the winter rape was completely finished, collections were continued from the spring rape. Most of the parasitoids were used in studies on their behavioural responses to oilseed rape volatiles (Jönsson et al., *in prep*). After use in experiments the parasitoids were stored until they were identified using the keys of Horstmann (1971). The relative abundance of female parasitoids was evaluated for days when the number of identified parasitoids where greater than ten.

Results and discussion

The most common species in the winter rape were *T. heterocerus* and *P. interstitialis*, while *P. morionellus* was the most abundant in spring rape (Figure 1). The same three species were also found to be the most abundant in an earlier study performed in the same area (Nilsson, 1985; Nilsson & Andreasson, 1987). Their temporal occurrence is also in agreement with the patterns found by Nilsson (1985).

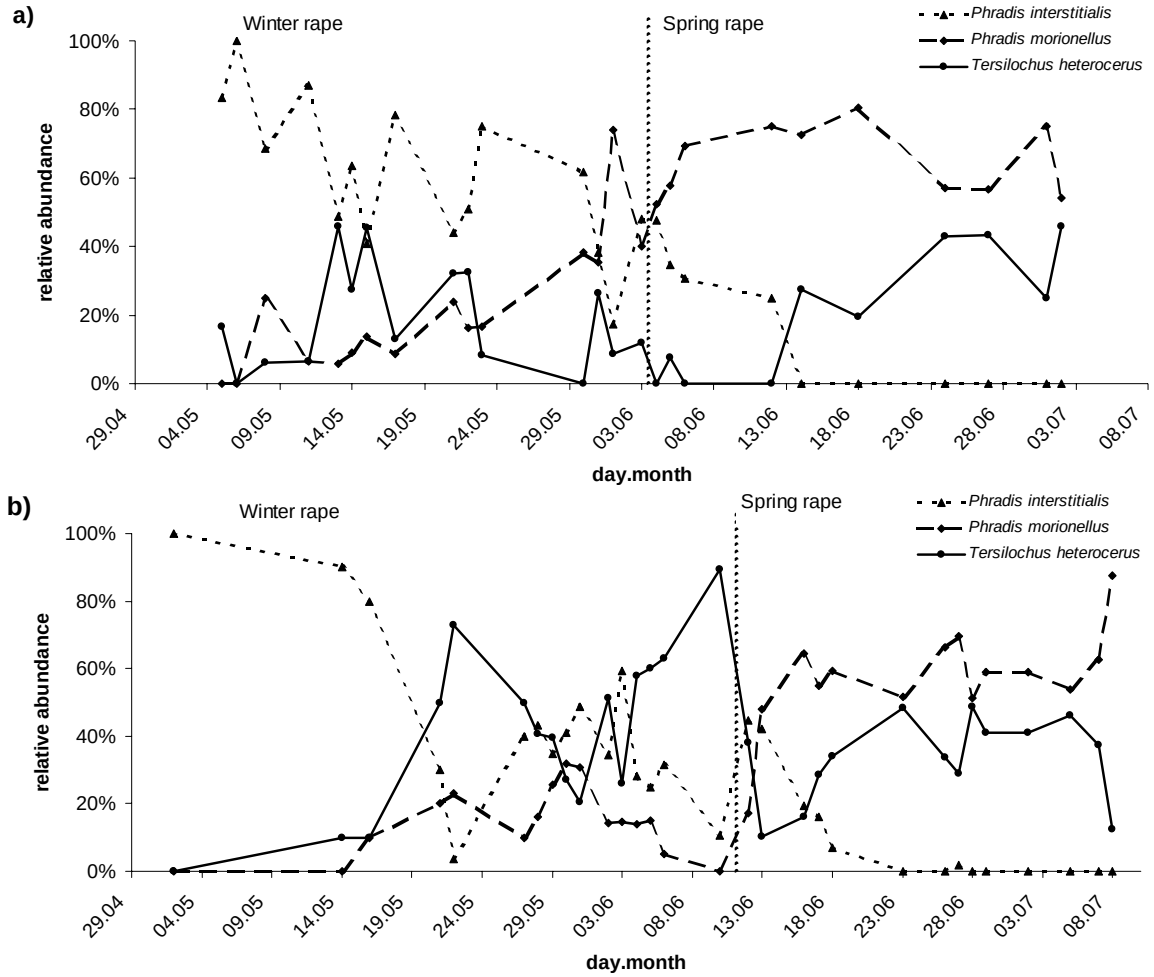

Figure 1. Relative abundance of three Ichneumonid parasitoids of pollen beetles collected in winter rape and spring rape. a) Parasitoids collected in 2002 b) Parasitoids collected in 2003. The vertical dotted line indicates when collection in winter rape ended and collection in spring rape started.

In 2002, the total number of collected and identified parasitoids was 355 in the winter rape and 210 in the spring rape. In 2003, the number of parasitoids caught from winter rape was 600 and the number from spring rape 591. Less than 1% of the collected female parasitoids were of other species than the described ichneumonids. Most of these were non-pollen beetle parasitoids of the genus *Tersilochus* or *Dacnusa* (Braconidae). Only a few *Diospilus capito* were found. A small, but higher proportion of *D. capito* were found in the earlier study by Nilsson & Andreasson (1987). Much higher proportions of *D. capito* have been observed in recent studies in Sweden (Billqvist & Ekbom, 2001; Haldén, 2004). The difference observed could be due to geographic variation, but in addition, is likely to be an effect of different collection techniques used (Haldén, 2004). In the study by Haldén (2004) the proportion of pollen beetle larvae in spring rape parasitised by *D. capito* was high, in spite of the small numbers of adult parasitoids that were caught with sweep nets.

The arrival of the first parasitoids and the development of the oilseed rape were a few days earlier in 2002 than in 2003. However, during these last days of April, almost all the parasitoids caught were males. In both years *P. interstitialis* was the most common parasitoid in the beginning of May, when the rape was still in the bud stage. Adults of *P. interstitialis* often arrive to the rape field before the other two species. Females of this species have, in opposite to the other two species, been observed to search the buds with their ovipositor and are able to oviposit in pollen beetle larvae inside the buds (Winfield, 1963; Nilsson, 2003). In 2002, *P. interstitialis* was the most abundant parasitoid in the winter rape while in 2003, the total number of *P. interstitialis* and *T. heterocerus* collected and identified were almost equal (252 and 256, respectively). Fluctuating abundance of the different species between years, as observed for *P. interstitialis* and *T. heterocerus*, are normal. Many different factors influence the number of parasitoids. One of the most important factors is the availability of pollen beetle larvae during the previous year (Nilsson, 2003). The most abundant species in the spring rape was *P. morionellus* but the percentage of *T. heterocerus* was relatively high compared to other investigations made in spring rape (Billqvist & Ekbom, 2001a; Haldén, 2004). It is possible that the southern location of the field is one explanation for this.

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Phenology and spatial distributions of *Dasineura brassicae* and its parasitoids in a crop of winter oilseed rape: implications for integrated pest management

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Abstract: The phenology and distributions of *Dasineura brassicae* and its parasitoids were studied in relation to the value of spatial and temporal information for the conservation of parasitoids in integrated pest management. Insects were sampled at 40 spatially-referenced points within a crop of winter oilseed rape and the following crop of winter wheat. In 1999, two generations of mature *D. brassicae* larvae were collected into water trays placed below the crop canopy, as they fell from rape pods to the soil for pupation. Insects emerging from *D. brassicae* larval cocoons in the soil were collected in emergence traps pre-diapause (1999) and post-diapause (2000). Spatial distributions were analysed and compared using Spatial Analysis by Distance IndicEs (SADIE). Only 7% of first generation larvae gave rise to emerging insects pre-diapause and 0.2% of first and second generation larvae to emerging insects post-diapause. Parasitoids comprised 42% and 49% of insects emerging from *D. brassicae* cocoons pre-diapause and post-diapause, respectively. *Omphale clypealis* was more abundant than *Platygaster subuliformis* in 1999. Pre-diapause, the start of emergence of both parasitoid species was coincident with the emergence of adult *D. brassicae* but the emergence of parasitoids was more prolonged. Emergence of *O. clypealis* post-diapause peaked a month later than either *D. brassicae* or *P. subuliformis*. All insects were markedly edge-distributed and spatially associated pre-diapause but only *O. clypealis* remained edge-distributed post-diapause. The implications for conservation management of parasitoids are discussed.

Key words: *Dasineura brassicae*, *Omphale clypealis*, *Platygaster subuliformis*, parasitoid, spatial distribution, phenology, integrated pest management.

Introduction

A succession of insect pests migrate from sites of diapause to colonise winter oilseed rape. Each is host to parasitoids which have considerable potential to exert control over their populations (Alford, 2003). However, parasitoids are likely to be vulnerable to insecticides, not only if applied for control of their own hosts but also when used to control other pests on the crop. The development of resistance to pyrethroids in the pollen beetle, *Meligethes aeneus* (Fab.), in Europe (Hansen, 2003) underlines the need for control strategies which optimise the role of biocontrol agents and minimise insecticide use (Williams *et al.*, 2003; Williams, 2004). Spatial and temporal information on the relative distributions of pests and parasitoids within crops is needed to underpin the development of targeted pest control and parasitoid conservation strategies (Murchie *et al.*, 1999b; Winder *et al.*, 1999; Ferguson *et al.*, 2000; Ferguson *et al.*, 2003). Recent studies have demonstrated that spatio-temporal patterns of pest infestation in oilseed rape can be complex and that insecticide application can be temporally targeted to conserve parasitoids (Murchie *et al.*, 1997; Ferguson *et al.*, 2003).

Dasineura brassicae, the brassica pod midge, is a major pest of winter oilseed rape in Europe (Alford *et al.*, 2003). It is multivoltine with two generations each year on winter rape (Williams *et al*., 1987). It lays its eggs into the pods and the larvae feed on the tissues of the pod walls, causing the pods to split prematurely, shedding seed. Mature larvae fall to the soil where they pupate. Most first generation larvae and a few second generation larvae emerge as adults the same year but a proportion of each generation will enter diapause for one or more winters (Williams *et al*., 1987). Two species of endoparasitoid attack the larvae of *D. brassicae*: *Omphale clypealis* (Hymenoptera, Eulophidae) and *Platygaster subuliformis* (Hymenoptera, Platygastridae) (Murchie *et al.*, 1999a; Williams, 2003). Each completes its development after the mature host larva has fallen to the soil. Both species are probably multivoltine like their host. Here, the spatial and temporal relationships between *D. brassicae* and its parasitoids, *O. clypealis* and *P. subuliformis*, in a winter oilseed rape crop are investigated in relation to the potential for conservation management of the parasitoids through changes in agronomic practices.

Materials and methods

All samples were taken from a 2.4 ha field sown with winter oilseed rape, *Brassica napus* (L.) (cv. Apex) in 1998/1999 and winter wheat in 1999/2000. No insecticides were applied after autumn 1998. Forty sample locations were defined on three concentric polygons and a central line at 3, 20, 40 and 58-66 m from the crop boundary (Fig. 2i). Mature *D. brassicae* larvae dropping from rape pods to pupate in the soil, were collected in trays (220x260 mm) containing water with detergent from 1 May to 19 July 1999 (when the crop was harvested). Emergence traps ($0.5m^2$) were placed at each sample location from 5 June to 19 July 1999 to record the pre-diapause emergence from the soil of second generation adult *D. brassicae* and parasitoids; they excluded further larvae from dropping into their catchment area and were emptied every four days. Post-diapause emergence of each species was recorded from 15 March to 2 August 2000, using the same traps emptied weekly.

Spatial patterns of insect counts were analysed using 'Spatial Analysis by Distance IndicEs' (Perry, 1998a; 1998b) to statistically define areas of clustering and gaps and to compare distributions. This technique allows the spatial characteristics of a single set of counts to be assessed by comparing the observed distribution to randomised permutations of the same counts amongst the sample locations. A set of local clustering indices, ν, is derived which indicates whether each sample unit falls within a cluster or a gap in the distribution of counts. Values of ν are positive in clusters and negative in gaps. The locations of clusters and gaps are illustrated by contour maps of the cluster indices drawn using 'Surfer' software (Surfer 8, Golden Software, Inc, Colorado, USA). Contours are interpolated by kriging.

Results

Mature *D. brassicae* larvae dropped from rape pods during every four-day sampling period from mid May 1999. There were two peaks of abundance probably representing two generations of larvae, the first from mid May to late June and the second from late June to mid July when the crop was harvested (Fig. 1a). Of the first generation larvae which dropped to the ground before emergence traps were in position, 6.8% gave rise to adult insects emerging the same year. Of these 42% were parasitoids, mostly *O. clypealis* (Table 1). Only 0.2% of all *D. brassicae* larvae of both 1999 generations gave rise to adults in 2000. Of these 49% were parasitoids, with similar numbers of *O. clypealis* and *P. subuliformis* (Table 1).

Table 1. Numbers of adult *D. brassicae* and parasitoids in emergence traps in 1999 and 2000

Adult emergence year	1999			2000		
Source population	1774 ± 197 larvae/ m^2 [a]			7342 ± 502 larvae/ m^2 [b]		
	insects / m^2 (mean±SEM)	% of source popn.	% of emerged adults	insects / m^2 (mean±SEM)	% of source popn.	% of emerged adults
D. brassicae adults	70.1 ± 8.6	4.0	58.2	6.70 ± 0.78	0.09	51.4
O. clypealis adults	45.0 ± 5.0	2.5	37.3	3.30 ± 0.67	0.04	25.3
P. subuliformis adults	5.45 ± 0.83	0.31	4.52	3.05 ± 0.46	0.04	23.4
Adults of all species	120.5 ± 13.1	6.8	100	13.1 ± 1.26	0.18	100

[a] *Mean ± SEM Dasineura brassicae larvae caught in water trays before 5 June 1999 when the emergence traps were placed in position.*

[b] *Mean ± SEM Dasineura brassicae larvae caught in water trays throughout 1999.*

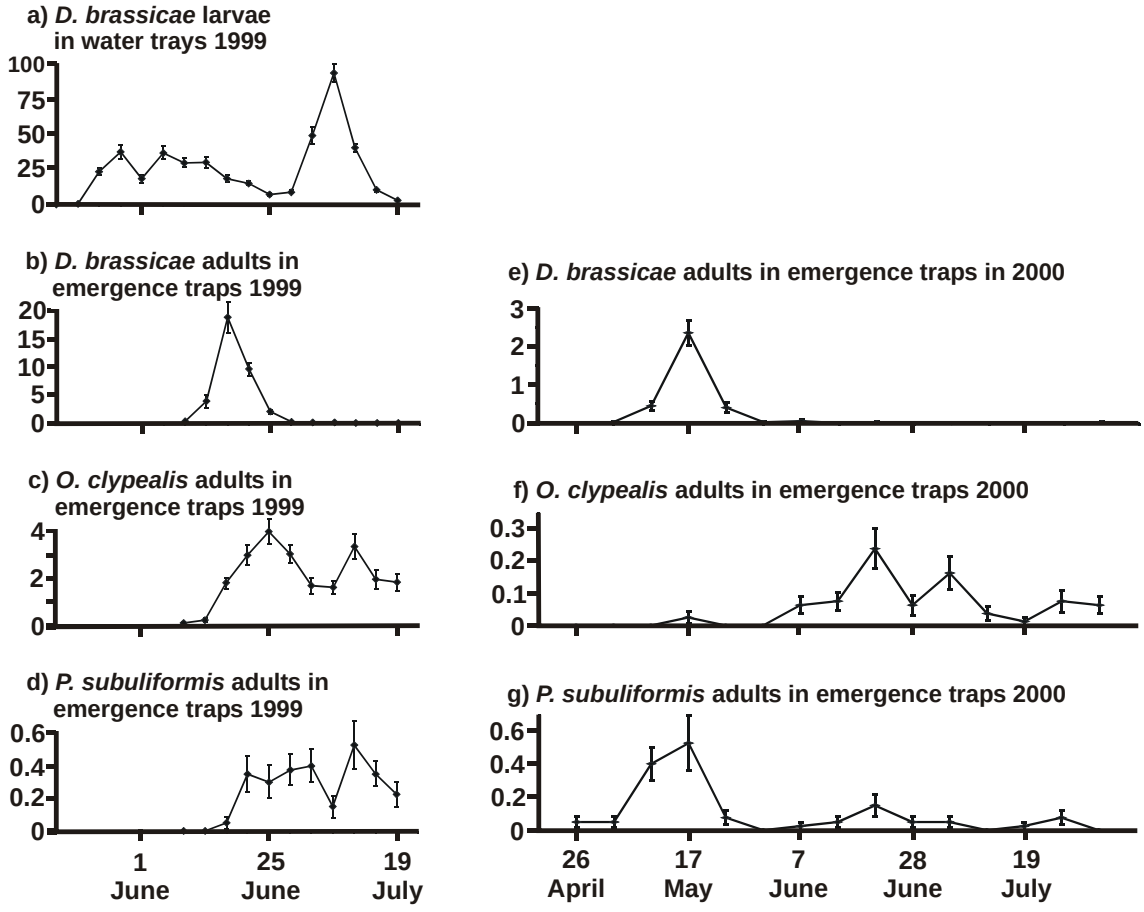

Figure 1. Phenology of emergence of *D. brassicae* larvae from pods in 1999 and *D. brassicae* and parasitoid adults from the soil in 1999 and 2000. Y axes: mean numbers of insects per water tray or emergence trap; error bars = 2 x SEM.

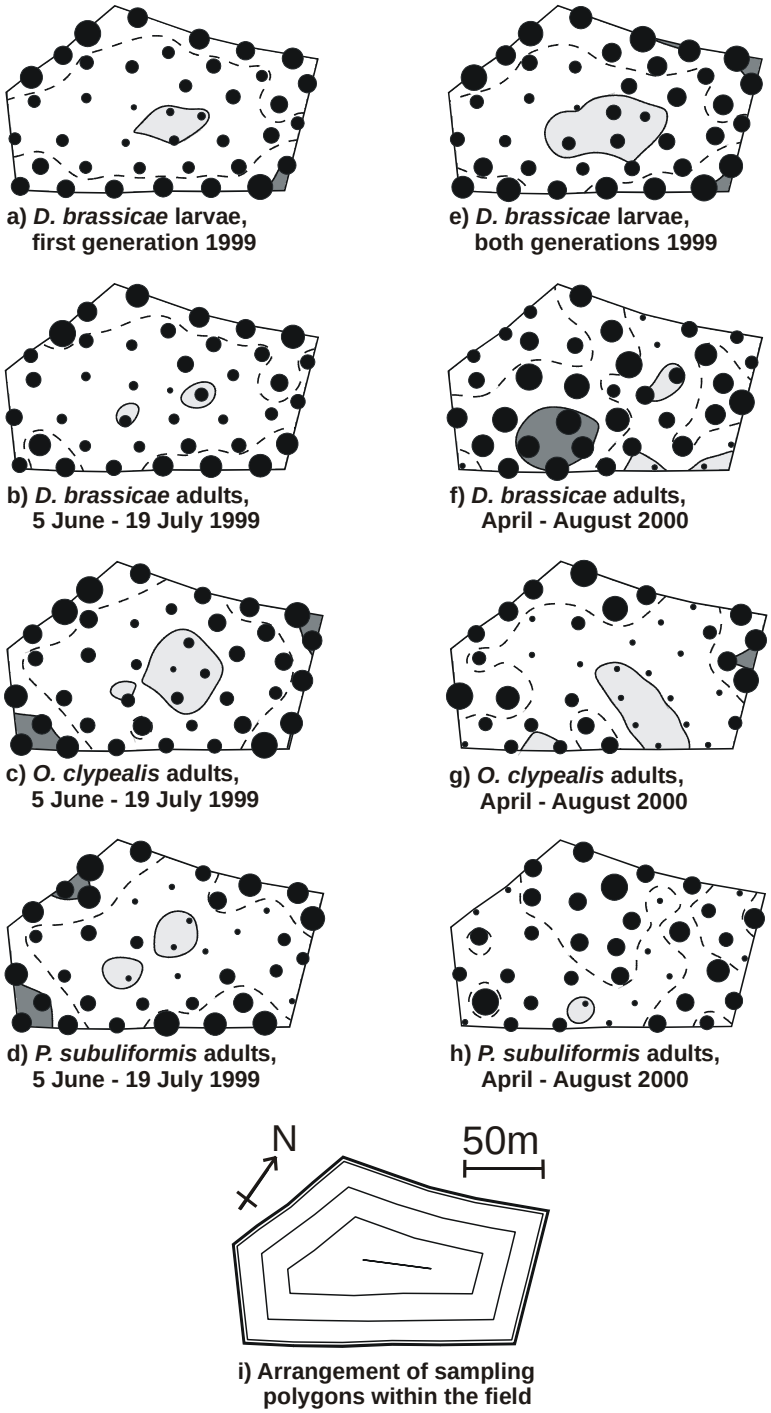

Figure 2. (a-h): Distributions of *D. brassicae* larvae in water trays in 1999 and *D. brassicae* and parasitoid adults in emergence traps in 1999 and 2000. Black discs represent the square root of the insect count at each site and are proportionally scaled between the minimum and maximum count for each map. Contours filled with pale and dark shading are areas identified by SADIE as gaps and clusters, respectively. Dashed lines are the zero contour for the SADIE cluster index, separating locations with tendencies towards gappiness or clustering. (i) Scale plan showing arrangement of sampling polygons within the field.

Almost all *D. brassicae* adults that emerged in 1999 did so over a 20-day period following the placement of the emergence traps, peaking on 17 June (Fig. 1b). Emergence of both parasitoid species was slightly later and continued until crop harvest (Fig. 1c & 1d). Post-diapause, the emergence of *D. brassicae* peaked on 17 May 2000, a month earlier in the year than in 1999, but it likewise occurred over a discrete three week period (Fig. 1e). The emergence of both parasitoids post-diapause again took place over a longer period than that of *D. brassicae* but, whereas peak emergence of *P. subuliformis* closely matched that of its host (Fig. 1f), the emergence of *O. clypealis* peaked a month later on 21 June (Fig. 1g).

Dasineura brassicae larvae and adults and *O. clypealis* adults were found at every sampling location; *P. subuliformis* adults were found at every location except one at the crop centre. The spatial distribution of *D. brassicae* larvae was strongly edge-distributed whether both generations were tested or the first generation alone (Figs. 2a & e). In each case, the zero contour of the SADIE cluster index separated regions of greater clustering and higher population at the edge from regions of greater gappiness and lower population in the centre; moreover significant clusters and gaps were identified by SADIE at the edge and centre, respectively. Pre-diapause, the spatial pattern of emerging adult *D. brassicae* appeared to closely match the pattern of the first generation larvae from which they derived, as did the patterns of emerging *O. clypealis* and *P. subuliformis* (Fig. 2a-d).

By contrast, the distribution of *D. brassicae* adults emerging post diapause showed no sign of edge-distribution, indeed most of the largest counts occurred well within the field and SADIE identified a cluster which extended towards the centre (Fig. 2f). *Platygaster subuliformis* adults emerging post diapause had also lost their edge-distribution but unlike their adult hosts they showed little sign of centre-distribution (Fig. 2h). Only adult *O. clypealis* retained their edge-distribution post diapause (Fig. 2g).

Discussion

Populations of D. brassicae *and parasitoids*

A population of *D. brassicae* became established across the experimental crop and was parasitised by both *O. clypealis* and *P. subuliformis* at almost every sample location. The proportion of insects emerging that were parasitoids was 42% pre-diapause and 49% post-diapause, high in comparison with earlier studies in the UK or elsewhere in Europe (Murchie, 1996; Williams, 2003). The proportion of parasitoids that were *O. clypealis* was 89% pre-diapause and 52% post-diapause, well in excess of the overall 4% reported on winter oilseed rape in the UK by Murchie (1996) who found *O. clypealis* to be more abundant on spring rape. It is not possible to infer true parasitism rates from the number of adults emerging.

Winter mortality

Only 6.8% of *D. brassicae* larvae gave rise to *D. brassicae* adults or to parasitoids pre-diapause and only 0.18% post-diapause. This compares with 27% and 16%, respectively, for field-collected, laboratory-reared larvae reported by Murchie (1996). Both the pest and its parasitoids probably therefore suffered significant levels of mortality while on the soil surface and in the soil, particularly during winter.

Predation by generalist epigaeic predators is a potentially significant mortality factor for larvae of *D. brassicae* that fall to the ground to pupate (Büchs & Nuss, 2000; Warner *et al.*, 2000; Büchs, 2003b). Axelsen (1992) estimated *D. brassicae* larvae suffered 53 - 73% mortality when on the soil surface, probably through predation. Pathogens almost certainly also contribute to mortality in the soil but their impact is little understood (Hokkanen *et al*.,

2003). Soil tillage has been identified as a significant cause of mortality for soil overwintering tersilochine parasitoids of other oilseed rape pests; ploughing can cause 50-80% losses or more (Nilsson, 1985; Klingenberg & Ulber, 1994; Nilsson, 2003). *Dasineura brassicae* and its parasitoids are probably similarly affected by cultivations during winter diapause. Extended diapause may be another cause of the apparent overwintering losses in both *D. brassicae* and its parasitoids. A proportion of *D. brassicae* may remain in diapause in the soil for more than one winter (Ankersmit, 1956; Buhl, 1960).

Pre-diapause phenology

There were two peaks in numbers of *D. brassicae* larvae dropping from rape pods, consistent with the presence of two generations on winter oilseed rape in Europe (Sylven, 1949; Buhl, 1960; Williams *et al.*, 1987). Pre-diapause, the collection of *D. brassicae* adults by emergence traps was complete within three weeks of trap deployment, when the traps excluded further larvae from reaching the soil. This agrees with the 11-23 day pupal development time reported in the UK (Williams *et al*., 1987). Given the extended period over which first generation *D. brassicae* larvae dropped from pods, the emergence of adults into traps was probably greatly truncated through the early exclusion of larvae. Pre-diapause emergence of adult *O. clypealis* and *P. subuliformis* was slightly later than their hosts, probably as their development is delayed until the host larva is mature (Williams, 2003). In contrast to their host, adult parasitoids of both species continued to emerge over a prolonged period until harvest. This may be a risk-spreading strategy which increases the chance that some individuals will coincide with the maximal availability of hosts.

Post-diapause phenology

The post-diapause emergence phenology of adult *D. brassicae* was unimodal, as reported by Williams *et al*. (1987), and reached a maximum on 17 May. This was matched very precisely by the peak in emergence of *P. subuliformis*, few of which emerged after mid May. The close coincidence of the post-diapause emergence phenology of *P. subuliformis* and its host accords with most other findings in Europe (Williams, 2003). By contrast, very few *O. clypealis* emerged in synchrony with the first generation of their host post-diapause, peak emergence occurring a month later.

Spatial distributions

Strong edge-distributions of *D. brassicae* infestation within fields have frequently been reported elsewhere (Ankersmit, 1956; Free & Williams, 1979; Ferguson *et al.*, 2003) and in this study were observed in both larval generations and in the distribution of second generation adults emerging pre-diapause. Not surprisingly, this was reflected in the distribution of parasitoids emerging from their hosts pre-diapause. However, there was a dramatic change towards centre-distribution amongst *D. brassicae* emerging post-diapause. This was reflected more weakly in the changing distribution of emerging *P. subuliformis* post-diapause whereas *O. clypealis* remained edge-distributed. These changes probably reflected soil-associated mortality factors which were themselves edge-distributed and acted more strongly over the long winter diapause than during the shorter pre-diapause pupal development, but had less impact on parasitoids.

Potential causes of changes in spatial distributions

Parasitoids were edge-distributed in this field but could reduce the edge-distribution of their host only if they showed a positive density dependent response to host abundance. There was no evidence for this in the pre-diapause emergence of parasitoids, nor for any change in the

distribution of *D. brassicae* adults emerging pre-diapause from a population which had been subject to parasitism. The activity of generalist predators, particularly Carabidae, is often more intense at the edges of fields (Büchs, 2003a) and soil at headlands is likely to be subject to more disruption and compaction during cultivation, causing increased injury to overwintering cocoons and obstructing emergence of adults in spring. However, it is not clear why either predators or soil conditions should be likely to impact less upon the parasitoids than their hosts.

Potential for conservation biocontrol

Temporal targeting of insecticides to avoid periods of parasitoid activity are a potentially valuable element of conservation biocontrol (Alford *et al.*, 1996; Murchie *et al.*, 1997; Williams, 2004). Overwintered *O. clypealis* emerged at a time when they would rarely be at risk from insecticide applications to winter rape. However, adult *P. subuliformis* emerged at mid flowering stage in May, coincident not only with the first host generation but also with the period when pyrethroid insecticides may be applied for the control of *Ceutorhynchus assimilis*. Temporal targeting is therefore not a viable option for conserving *P. subuliformis.* The close spatial coincidence in the rape crop of both parasitoid species and *D. brassicae* also precludes any possibility of conserving the parasitoids by spatial targeting of insecticide treatments towards their host.

Further research funded by the EU project MASTER (Williams *et al.*, 2003) is investigating whether reduced tillage for conservation of parasitoids could have a beneficial effect on control of *D. brassicae*. The success of reduced tillage would depend on the relative effect upon the population dynamics of each species. Research is also needed to characterise the major causes of overwintering mortality in the soil, particularly those edge-distributed factors that influence *D. brassicae* more strongly than its parasitoids. Such information could be valuable in adapting crop husbandry practices to maximise biocontrol. There may be potential for the integrated management of *D. brassicae* by conservation of both parasitoids and predators close to the crop boundary using a combination of reduced tillage and field margin management (Warner *et al.*, 2000).

Acknowledgements

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Verification of protective sowing ability to concentrate insect pests and their parasitoids around oilseed rape field

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Abstract: Some insect pests do not attack the whole oilseed rape field evenly but prefer especially a margin of the field. The reduction of insect pest population due to the increasing distance from the field margin is called "margin effect". The lines of plant attractants against insect pests were sown around the margins of oilseed rape fields. Turnip rape (*Brassica rapa* L.), variety Rex, an extra early variety of oilseed rape – Prestol and mixture of Turnip rape, Prestol and spring oilseed rape were used as a plant attractant. The observed results of the work show, that the first flight of *M. aeneus* could be considerably trapped by a protective sowing. The protective sowing is also very attractive for *C. assimilis*. The number of beetles was the highest in the protective sowing in all experiments. The attractivity could be partly suggested also for *D. brassicae*. The results prove that the *M. aeneus* and *C. assimilis* are more attracted by turnip rape plants than by early varieties of oilseed rape. The influence of protective sowing on the crop infestation by *C. napi* and *C. pallidactylus* was not proved. The method of protective sowings could contribute to the increase of insect pest parasitoids as well. Detected were mostly the adult endoparasitoids from *Tersilochus* spp. (Hymenoptera: Ichneumonidae). A significantly higher number of the parasitoids in the protective sowing than in the main crop was prooved. Based on the analysis of the *M. aeneus* larvae, it could be stated, that the level of parasitism through *Tersilochus* spp. ranged between 75 – 100 % in the protective sowing and between 60 – 100 % in the main crop. There was an evident dependence between the high density of the *M. aeneus* beetles and parallel high density of their parasitoids and the level of parasitised larvae.

Key words: protective sowing, winter oilseed rape, turnip rape, *Meligethes aeneus*, *Ceutorhynchus asssimilis*, *Dasineura brassicae*, stem weevils, parasitoids, *Tersilochus* spp.

Introduction

The increase of winter oilseed rape (*Brassica napus*, L.) fields in the Czech Republic caused the higher occurrence of insect pests. The stem weevils (*Ceutorhynchus napi*, *Ceutorhynchus pallidactylus*), the pollen beetle (*Meligethes aeneus*), the cabbage seed weevil (*Ceutorhynchus assimilis*) and the brassica pod midge (*Dasineura brassicae*) settle down in the plants during spring period and are included in the cardinal insect pests. Some of them do not attack the whole oilseed rape field evenly but prefer especially a margin of the field. The reduction of insect pest population due to the increasing distance from the field margin is called "margin effect". ŠEDIVÝ (1982); (VAŠÁK, 1988); PAUL, RAWLINSON (1992) referred about the treatment of oilseed rape field margins against insect pests such as *M. aeneus* and others, because of their high density right on the margin.

In the intensive agricultural production, multiple and whole surface area sprays are used as a usual way of protection against these insect pests. This situation is not optimal both from the environmental as well as the economical point of view.

The aims of this work were:

To verify the ability of plant attractant lines sown around the margins of oilseed rape fields to concentrate insect pests and their parasitoids.

To investigate various degree of attractivity of the selected Brassica species for important insect pests.

To enable essential reduction of chemical input into a production and the reduction of expenses without increased level of oilseed rape damages.

Material and methods

The lines of plant attractants against insect pests were sown around the margins of oilseed rape fields at two different localities. Turnip rape (*Brassica rapa* L.) variety Rex was used as a plant attractant. This crop has the similar seed composition and growing conditions as winter oilseed rape. In 1999 and 2000 the experiment was extended by an extra early variety of winter oilseed rape variety Prestol and mixture of turnip rape, Prestol and spring oilseed rape as well (Table 1). "Natural method"- without a treatment against insect pests, "current method" by means of 2-3 doses of insecticide spray and the method with spraying specially directed only on the protective sowings were compared (Figure 1). The flight of insect pests was followed in yellow trap dishes located in the protective sowings and in the main crop.

Table 1. Overview of experimental variants.

Experimental year	Variant **A**/ without sprays (control)	**B**/ whole area sprays	**C**/ sprays only on the sowings	Composition of protective sowing
1997/98	+	+	-	100% of winter turnip rape, variety Rex
1998/99	+	+	+	100% early winter oilseed rape, variety Prestol
1999/00	+	+	+	the mixture: 50% Rex, 25% Prestol, 25% spring oilseed rape

Figure 1. The schematic figuration of experimental variants grounded on field with protective sowing. Var A, B, C – see table 1, width of protective sowing - 9m, width of variant A and C (cross-sections of field) 18m. (*) means without insecticide treatments.

The frequency of adult pests on the main racemes of 4 x 25 plants (M. aeneus, C. assimilis) was observed in protective sowings and in different distances on the "cross-section" of experimental fields (25 m, 50 m and the middle of field 100 – 150 m), (Fig. 1). In case of sowing mixture the pests on turnip rape were collected separately. In addition, the percentage of invaded stems and the level of damage by larvae of C. napi and C. pallidactylus were also calculated. Moreover, the occurrence of pest's natural enemies was evaluated (Table 2).

Table 2. Overview of factors observed on experimental fields.

Observed factor	**1998**	**1999**	**2000**
occurence of pests of generative parts	+[1)]	+	+
occurence of parasitoids	+[1)]	+	+
occurence of pollen beetle larvae and level of their parasitization	-	+	+
damage by stem weevils larvae	+	+	+
% damage of generative parts	+[2)]	+	+
occurence of fungal diseases (*Phoma lingam*)	-	+[3)]	+

1) *Collection only by „flicking off" –without entomological sweep (seed midge not trapped, parasitoids only in part)*
2) *the number of siliques and reduction of pods not evaluated*
3) *evaluated only orientationally*

The quantity of pests in protective sowing and in different distances on the "cross-section" of experimental fields were assessed in three different stages of growth related to protective sowing – yelow buds, begin and full bloom. The numbers of pests from variants without an insecticide treatment (a) by their highest invasion were used. The numbers were transformed to a percentage coefficient. As the value of 100% was always expressed the number of pests in the protective sowing. The file of trials with turnip rape (including sowing mixture) in protective sowing (2 years, 4 localities) and trials with only extra early oilseed rape in protective sowing (1 years, 2 localities) were evaluated separately.

Results and discussion

Occurrence of M. aeneus *and* C. assimilis

The results of experiments show, that protective sowing evidently reduce the number of pollen beetle and cabbage seed weevil in the main growth, where the threshold level generally is not exceeded. By both of the pests we suggested a significant difference between the number of adults in the protective sowing and the number inside of the main growth in all intake distances on the "cross section" of experimental field (Figure 2-3, 4-5). The differences between the pest numbers in the depths of 25 m, 50 m and in the middle of the field proved as insignificant.

This fact coresponds with the results of some previous studies (BEZECNÁ, VAŠÁK, 1988; FÁBRY et al., 1992; VAŠÁK et al., 1997). The authors referred the use of erucaacidless turnip rape variety Rex, that has the same growing conditions as oilseed rape and is about 10 – 14 days earlier. However, they restrict the use of protective sowings to the fields over 20 ha, well balanced land configuration and note about 30 % lower yield by the turnip rape.

Nevertheless, from our experiments ensue that by using of the protective sowing mixture (the turnip rape, early winter oilseed rape and spring rape) and the sowing width about 6-9 m, which shows to be sufficient, the yield loss significant decreases (the average yield of the mixture = 3,15 t ha^{-1}). The results with the sowing mixture show, that the Meligethes spp. prefer the turnip rape rather than earlier and more developed spring rape within the first high invasions of beetles in middle of April. This fact deconstruct the idea of dominant influence of the yellow colour by the pests attraction. Several days after, the spring rape comes into bloom at first, which increased her attractivity because of the easier accessibility of the pollen.

The recent studies refer that the oilseed rape and the other Brassica species have a different composition and production of some secondary plant metabolites. These metabolites arised especially from stress situations – e.g. damage by the pests etc. The volatile metabolites of glucosinolates decomposition - isothiokyanates release the plant tissues and attract the pests (BLIGHT et al., 1995, BARTLET et al., 1997).

Several days later increased the preference of the earliest spring rape by *C. assimilis* and *D. brassicae* in regard with the faster development and earlier formation of the young pods. This is the reason for using the very early spring rape in the protective sowing mixture. We detected a higher percentage of the damaged pods by the spring rape than by the turnip rape.

The use of the mixture of winter turnip rape with the winter or spring rape as a trap crop for the oilseed rape pests is referred for example by BÜCHI (1995) and NILSSON (1996). The protective 5 - 6 m wide stripes sowed at the circumference of the rape field showed an insufficient effect against stem weevils (*C. napi*, C. pallidactylus) however they have concentrated the population of the pollen beetle from 28,4 to 80,3 % in the first 7 - 10 days of invasions. They also enunciate the preference of the turnip rape before the rape by the pollen beetle. BÜCHI, (1990; in: BÜCHI, 1995) mentions a higher number of larvae of cabbage stem flea beetle (Psylliodes chrysocephala) and also the number of leaves feedings at the turnip rape than at the oilseed rape. BUNTIN (1998) finds the use of the in winter sowed spring rape as a trap crop that blooms 2 - 3 weeks before the main crop. The adults of *C. assimilis* were reduced in the course of blooming to about 60 to 80 %. The larvae in the pods had been reduced about 60 %. He notes the effectiveness of this system particularly by a low population of pests.

***Occurrence of parasitoids from* Tersilochus spp.**

The method of protective sowings could contribute to the increase of pest parasitoids as well. A numerous population of the parasitoids was trapped during the full blooming of the growth. Detected were mostly the adult endoparasitoids from *Tersilochus* spp. (Hymenoptera: Ichneumonidae).

This fact is consistent with the detection of Šedivý (1983), that insect from Hymenoptera order (family Ichneumonidae, subfamily Tersilochinae) are the most frequented parasitoids of the insect pests attacking the winter oilseed rape. Šedivý found a considerable variability in various localities even in the course of one year. A significantly higher number of the parasitoids in the protective sowing than in the main growth were proofed (Figure 6 - 7). This confirms the cumulation trend of parasitoids in protective sowings in a tight correlation with the cumulation of the pests, on which they parasitize.

Based on the analysis of the *M. aeneus* larvae it could be stated, that the level of parasitism through *Tersilochus* spp. ranged between 75 – 100 % in the protective sowing and between 60 – 100 % in the main growth. There was an evident dependence between the high density of the *M. aeneus* and parallel high density of their parasitoids and the level of parasitised larvae. This corresponds with Šedivý (1983), who found a correlation between the parasitism rates and the concentration of the *M. aeneus* larvae on the plants. The level of parasitism was higher in the years of the maximum pest appearance and in the following

years. The amount of larvae attacked by parasitoids was higher in distance up to 25 m from field margins. Inside the main growth – in 50 m and in the middle of field - the level of parasitism was lower nearly without differences (Fig. 8). The possibility of a strong decimation of the parasitoids population by insecticides applied in March and April is noted for example by ŠEDIVÝ (1983) and WILIAMS, MURCHIE (1995). Significantly lower numbers of parasitoids in the experimental growths with insecticide treatment were found. However, it is not possible to define exactly whether the population was decreased directly by the insecticide treatment or undirectly by decreasing of the potential host density.

***Occurrence of* C. napi *and* C. pallidactylus**

High invasions of stem weevils (*C. napi, C. pallidactylus*) were observed at the end of March at about 9 °C. 10-15 adults were found in yellow water traps. In this period, both the protective sowing and main crop were in the covered buds stage with a little difference of development, however. Therefore, the protective sowing could not still act as "yellow" or "odour" attractant for the earliest insect pests. The stem weevils were not attracted; neither by turnip rape, nor by mixture of turnip rape, early winter rape and spring rape.

In the "natural method" without treatments, a high percentage of stems (70 – 100 %) attacked by the stem weevils in the whole area of growth was found (Figure 9). On the contrary, the growth treated by Nurelle D (Pyrethroid + Organophosphate) approved a significant effect of sprays against this pest. The high level of injury corresponds with the results of JANKOWSKI, BUDZYŇSKI (1997). They indicate 88% of injured stems by chemical untreated growth. The insecticide application decreased the infestation more than thrice.

Poor effectiveness of protective sowings which included 5 – 20 % of turnip rape against stem weevils was mentioned also by BÜCHI (1995). In view of the insufficient effectiveness of protective sowing and high harmfulness of the stem weevils proves as suitable to keep the first insecticide whole area treatment in case of high invasion. The advantage of this treatment could be the residual leverage on first invasions of pollen beetle.

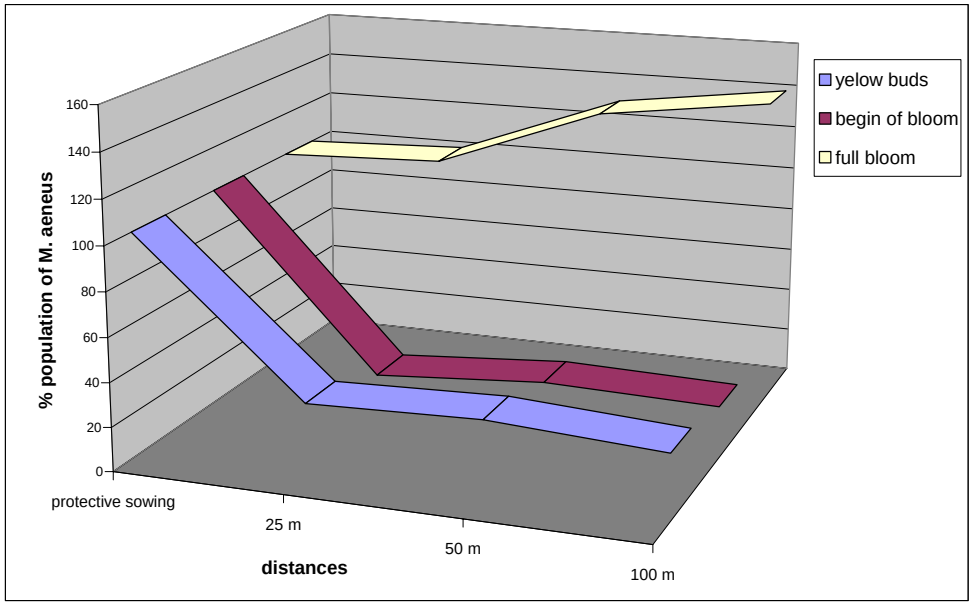

Figure 2. The average numerousness of *M. aeneus* population (%), results from 2 years (4 localities), protective sowings with turnip rape. Growth stage related to protective sowing.

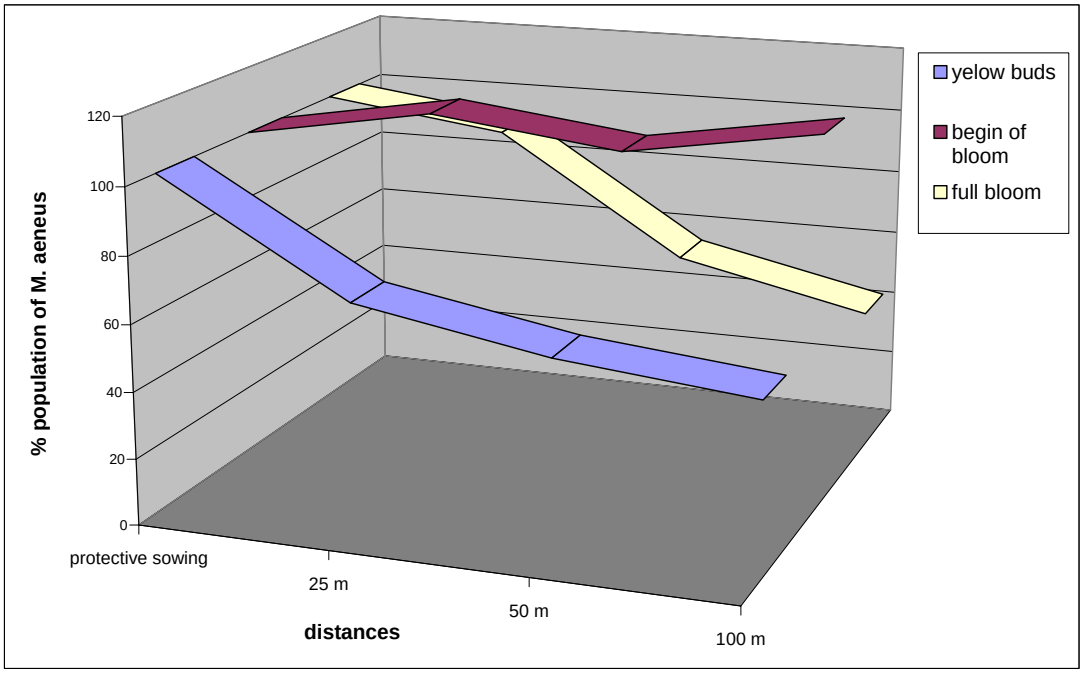

Figure 3. The average numerousness of *M. aeneus* population (%), results from 1 year (2 localities), protective sowings with very early oilseed rape. Growth stage related to protective sowing.

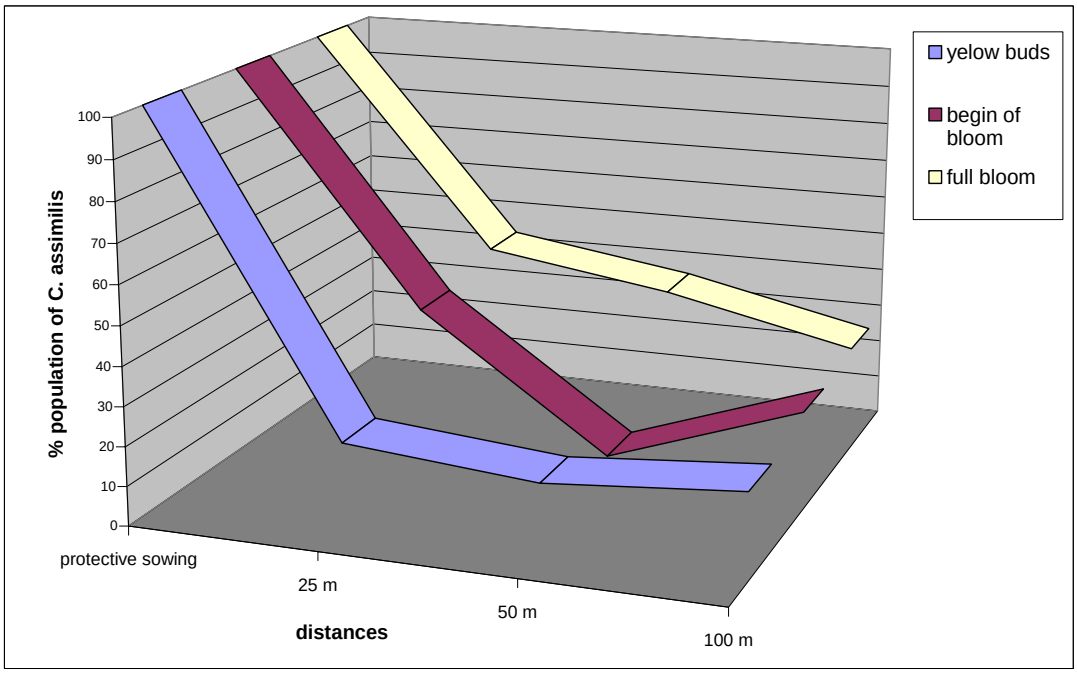

Figure 4. The average numerousness of *C. assimilis* population (%), results from 2 years (4 localities), protective sowings with turnip rape. Growth stage related to protective sowing.

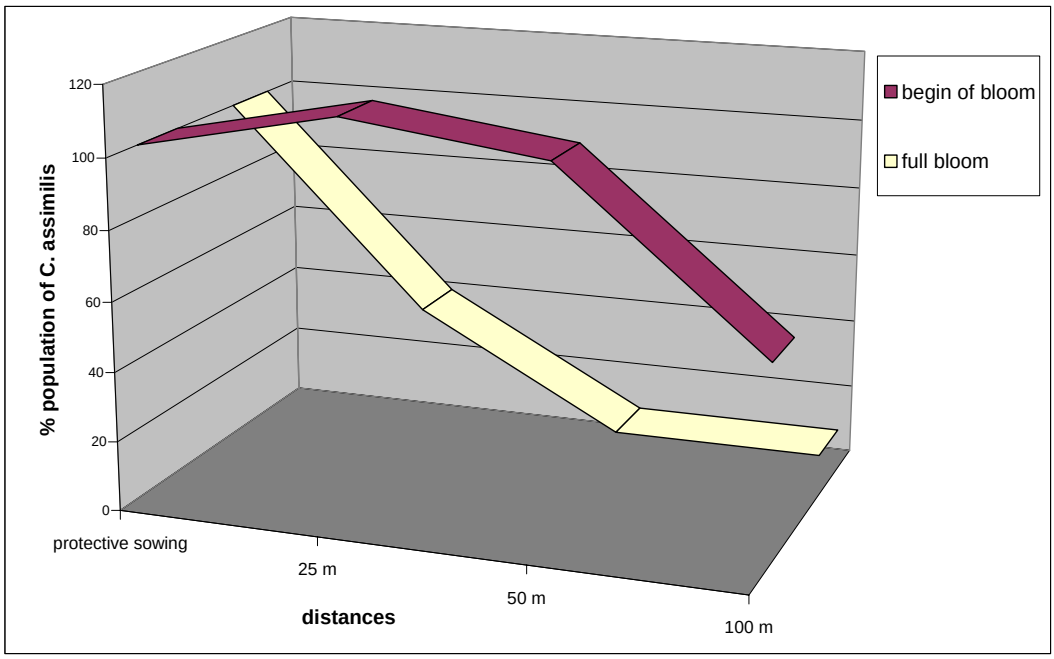

Figure 5. The average numerousness of *C. assimilis* population (%), results from 1 year (2 localities), protective sowings with very early oilseed rape. Growth stage related to protective sowing.

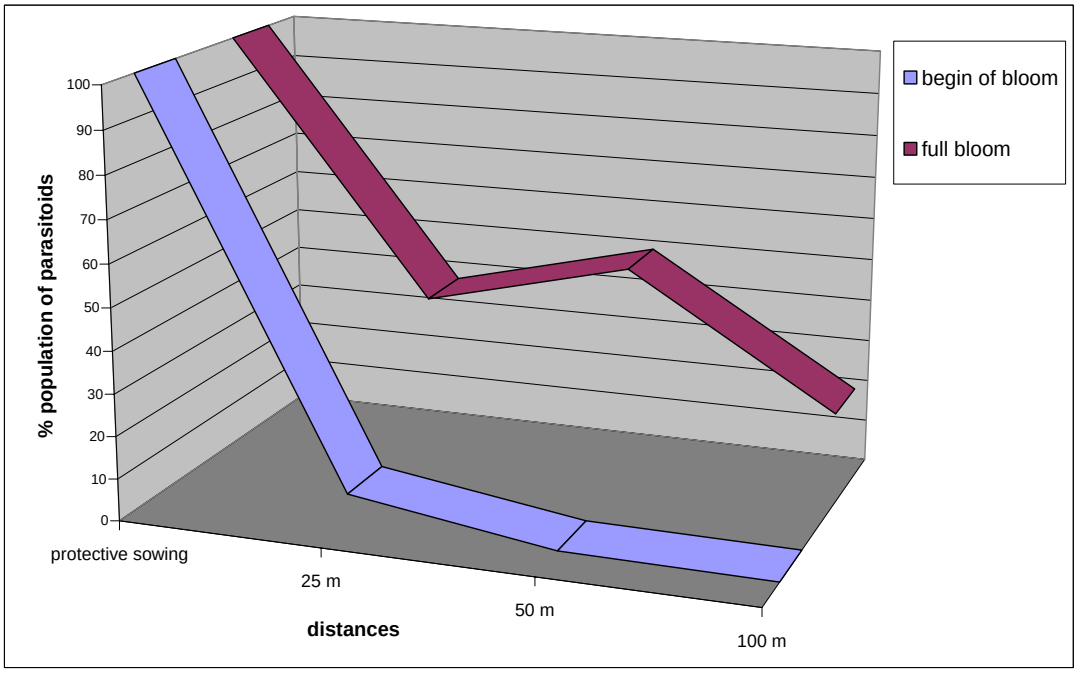

Figure 6. The average numerousness of parasitoids population (%), results from 2 years (4 localities), protective sowings with turnip rape. Growth stage related to protective sowing.

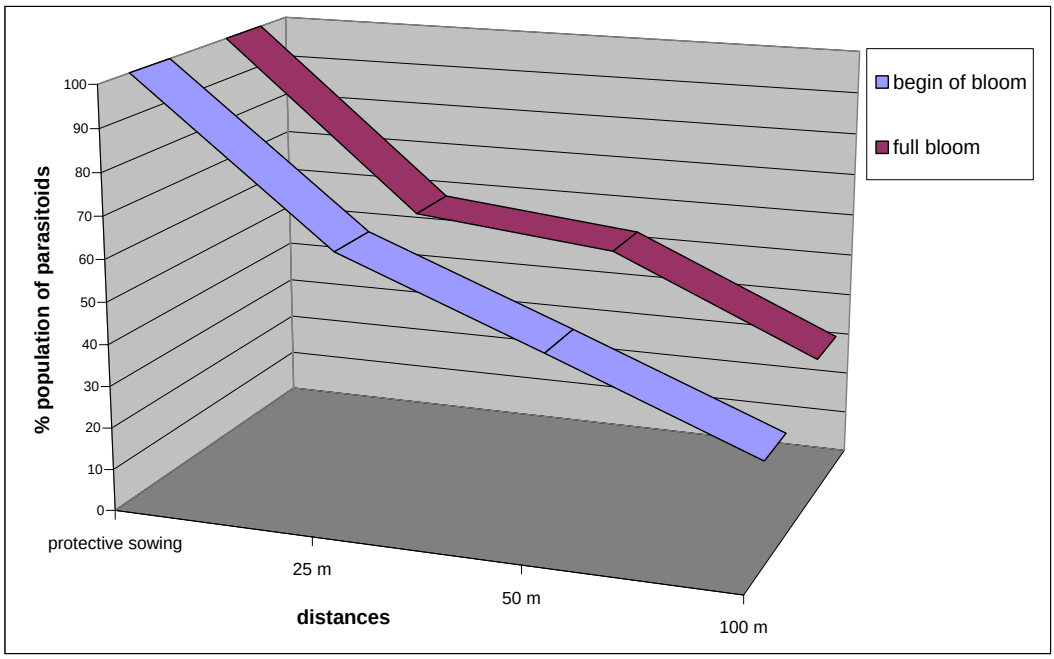

Figure 7. The average numerousness of parasitoids population (%), results from 1 year (2 localities), protective sowings with very early oilseed rape. Growth stage related to protective sowing.

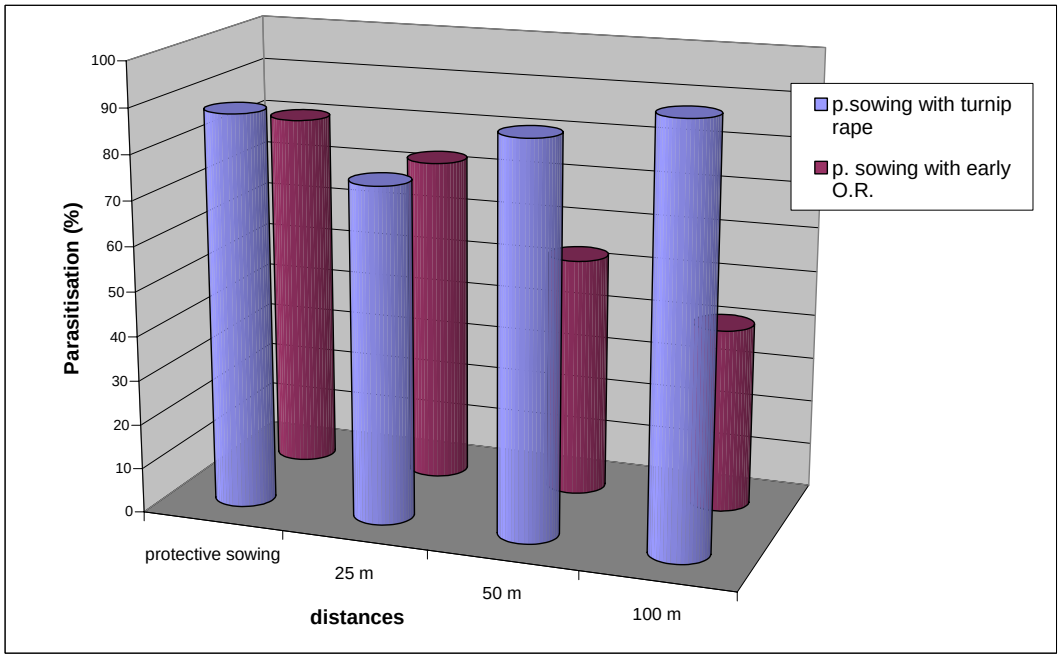

Figure 8. Infestation of *M. aeneus* larvae by parasitoids from *Tersilochus* spp.

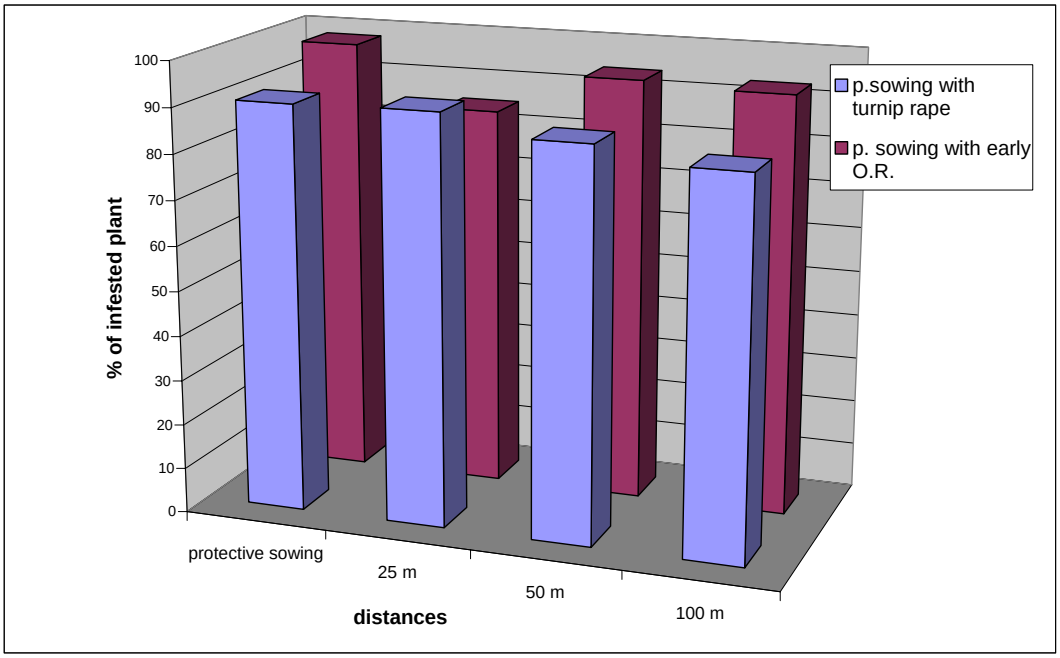

Figure 9. Infestation of plants by stem weevils larvae (*C. napi, C. pallidactylus*).

Conclusions

The observed results of this work show, that the first flight of *M. aeneus* could be considerably trapped by a protective sowing. There were more than 70% of adults trapped on the protective sowing. The protective sowing is also very attractive for *C. assimilis*. The number of beetles was about 80% higher in the protective sowing than in the main growth. The attractivity could be partly suggested also for *D. brassicae*. Because of the high density of the pests, the population of their parasitoids on the protective sowing is densely concetrated as well, ca about 50-70% more than in the main growth. A high level of parasitism of *M. aeneus* larvae (75-100%) was detected.

The presence of turnip rape in protective sowing is essential and it is suitable to mix it with spring rape and early winter oilseed rape. The results suggest that the pests are preferably attracted by specific host plant odour more than by the yellow colour of flowers. The distinct odour of turnip rape is based on a high glucosinolate content in generative tissues. That could explain a higher attractivity of turnip rape for pollen beetle in comparison with an early flowering rapeseed variety. Spring rape is the earliest in development of pods, which are suitable for eggs laying. Therefore, the spring rape is more attacked by *C. asssimilis* and *D. brassicae*.

The influence of protective sowing on the growth infestation by stem weevils (*C. napi, C. pallidactylus*) was not proved. Furthermore, there is a tendency to state the positive correlation between plant injury by stem wevils larvae and the dissemination of fungal diseases (*Leptosphaeria maculans*).

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Rearing and identification of the larval parasitoids of *Psylliodes chrysocephala* and *Ceutorhynchus pallidactylus* from field-collected specimens

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Abstract: *Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae) (cabbage stem flea beetle) and *Ceutorhynchus pallidactylus* (Marsh.) (Coleoptera: Curculionidae) (cabbage stem weevil) are two major stem-mining pests of oilseed rape (*Brassica napus*) in the UK. Little information is available on the occurrence, importance and efficiency of parasitoids in the biocontrol of these pests, due to sampling difficulties and inadequate knowledge on rearing and identification methods for the adults. In this study, we developed an efficient method of rearing the larval parasitoids of *P. chrysocephala* and to a lesser extent, *C. pallidactylus* from field-collected specimens in order to identify the active larval parasitoids of these pests. Plant samples were collected in 2002 and 2003 from unsprayed winter oilseed rape crops at ~10-day intervals from mid-April until July. The samples were kept in cages in an outdoor insectary and the pest larvae were collected as they left naturally from the plant stems when ready to pupate. The larvae were identified to species and put into either 'pot emergence trap' or 'corked tube' containers containing sterilised soil, then kept under natural environmental conditions in the insectary. The adult pests were collected and counted as they emerged. The soil of the containers was then examined and parasitoid cocoons sorted from it, collected and counted. The cocoons were dissected and the pre-emergence adult parasitoids were removed and identified. Adding 20% sharp sand to the soil increased the proportion of parasitoid cocoons found and reduced mortality in both containers. More parasitoid cocoons were found and lower mortality occurred in corked tubes than pot emergence traps in both years. These results suggest that the addition of sand to the soil and the use of corked tube containers contributed to the rearing success of the parasitoids by providing better conditions for their development. *Tersilochus microgaster* Szép. and *T. obscurator* Aub. (Hymenoptera: Ichneumonidae) were reared from *P. chrysocephala* and *C. pallidactylus* larvae, respectively. These parasitoids were identified for the first time in the UK as active parasitoids of these pests.

Key words: rearing, parasitoid, oilseed rape, *Psylliodes chrysocephala, Ceutorhynchus pallidactylus*, *Tersilochus microgaster*, *Tersilochus obscurator*, stem-mining pest.

Introduction

Although there have been many studies on parasitoids of oilseed rape (*Brassica napus* L.) (Brassicaceae) pests, particularly in Europe, information on their rearing and identification is incomplete. This information is required for biocontrol studies because, in order to elucidate which parasitoids attack which hosts, it is necessary to collect the pest larvae in question, rear them in controlled conditions and identify the emerging adult parasitoids. Sampling, trapping and rearing methods for oilseed rape pests, and a new key to the identification of the hymenopterous parasitoids associated with oilseed rape pests have recently been published (Vidal, 2003; Williams et al., 2003). However, as pointed out by these authors, there are still many unresolved problems associated with the rearing and identification of the parasitoids of oilseed rape pests, particularly the stem-mining pests.

Psylliodes chrysocephala (L.) (Coleoptera: Chrysomelidae) (cabbage stem flea beetle) and *Ceutorhynchus pallidactylus* (Marsham), syn. *C. quadridens* (Panzer) (Coleoptera: Curculionidae) (cabbage stem weevil) are stem-mining pests of oilseed rape in the UK (Alford et. al., 2003). They are univoltine with three larval instars. The larvae feed inside the leaves (petioles and veins) and stem of the plant causing damage (Graham and Gould, 1980; Cox, 1998; Alford *et al.*, 2003).

The larvae of oilseed rape stem-mining pests are attacked by parasitoid species belonging to three hymenopteran families: Ichneumonidae, Braconidae and Pteromalidae. The main larval parasitoids belong to the genus *Tersilochus* Holmgren (Ichneumonidae: Tersilochinae) (Ulber, 2003; Ulber and Williams, 2003). *Tersilochus* parasitoids oviposit into their host larvae within plant petioles or stems in the spring. Although the parasitoid egg hatches within the host larva, most of the parasitoid's larval development occurs within the prepupal stage of the host in the soil in summer. The parasitoid larva spins a cocoon then pupates. The adult wasp remains in diapause inside the cocoon and emerges the following spring (Ulber, 2000).

In continental European studies the following Tersilochinae have been found to parasitize oilseed rape stem-mining pest larvae: *Tersilochus tripartitus* Brischk, which attacks the larvae of *P. chrysocephala* and *C. pallidactylus* (Jourdheuil, 1960), *Tersilochus microgaster* (Szépligeti) which attacks *P. chrysocephala* larvae (Nitzsche, 1998), and *Tersilochus obscurator* Aubert and *Tersilochus exilis* Holmgren which both attack larvae of *C. pallidactylus* (Jourdheuil, 1960; Herrström, 1964; Ulber, 2000). However, it is not known which of these species are active parasitoids in the UK. One potential reason for the scarcity of information on parasitoids of stem-mining pests of oilseed rape is the difficulty in collecting adult parasitoids of the pests in the field. The phenology and emergence time of the parasitoids is little understood, so finding the correct time for sampling is difficult. In addition, the adult parasitoids usually occur at a low level within the plant canopy, where they search for stem-mining pest larvae (Barari et al., unpublished data) hence, collecting them by sweep-netting is not feasible. Therefore, to collect adult parasitoids and to ensure that they are active parasitoids of *P. chrysocephala* or *C. pallidactylus* larvae, we reared the larval parasitoids of the pests from field-collected hosts.

In continental Europe, there have been a few investigations on rearing the larval parasitoids of *P. chrysocephala* and *C. pallidactylus* infesting oilseed rape (Klingenberg and Ulber, 1994; Klukowski and Kelm, 2000; Kraus and Kromp, 2002). In all three studies, the pest larvae were dissected from plants collected in the field and then reared in plastic boxes containing sterilised moistened soil for pupation. However, Klingenberg and Ulber (1994) found that the parasitism rate of the fully developed pest larvae that leave the plant stems naturally when ready to pupate is higher than those collected through plant dissection. Therefore, to improve our chances of obtaining parasitized larvae, we aimed to develop more natural methods than used in previous studies to rear larval parasitoids of *P. chrysocephala* and *C. pallidactylus* in order to identify the active larval parasitoids of these pests in the UK.

Materials and methods

Plant sample collection

Oilseed rape (*B. napus* and *Brassica rapa*) stems damaged by the pest larvae were collected in 2002 and 2003 from crops that had not been treated with insecticide, grown on Rothamsted Farm, Harpenden, Hertfordshire, UK., at approximately 10-day intervals from mid-April until the crop was desiccated in July. This time period covered the whole period of adult parasitoid activity in the field (Barari et al., unpublished). Plants showing symptoms of stem-mining pest

damage (exit holes on stems and visible tunnelling damage to stems and petioles) were collected. The buds, flowers and pods were removed from the plants in the field to eliminate the larvae of other coleopteran pests e.g. cabbage seed weevil, *Ceutorhynchus assimilis*; only the stems were kept to capture the mature stem-mining pest larvae as they exited the stems, ready to pupate.

Pest larvae collection

The plant samples were taken to an outdoor insectary and kept in cages for one week. The insectary was protected from direct sunlight and had two sides open to the outside that were covered with net. Six cages were used to collect the pest larvae from plant samples. Each cage was 160 cm high, 50 cm long and 50 cm wide and had net sides with a plexiglass door. The cage had a drawer underneath which was separated from the main part of the cage by a metal grid. Mature larvae ready to pupate exited the stems and dropped down into the drawers. These were covered on the inside with a black plastic sheet to make the pest larvae more visible. The drawers were checked daily and any larvae removed using a moistened paint brush. They were identified to species (*P. chrysocephala* or *C. pallidactylus*) using the key by Alford et al., (2003) and put into either 'pot emergence trap' or 'corked tube' containers. The pot emergence traps (Figure 1) were each made of a plastic flower pot (13.5 cm diameter, 12.5 cm deep), on the top of which was a metal frame (14 cm height) topped with a plastic end screw connector. The frames and the top of the flower pot were covered with black tulle to prevent any emerging insect from escaping. Emerged insects were collected from a transparent inverted plastic container (8.5 cm diameter, 10.5 cm deep) with a screw-on lid. An open-ended sterilin tube connected the container via a screw end to the top of the metal frame. The corked tubes (Figure 2) were glass specimen tubes (2.4 cm diameter, 7.2 cm deep) with cork stoppers (VWR International Ltd., Lutterworth, Leicestershire, UK). Both pot emergence traps and corked tubes were half-filled with soil (collected from an oilseed rape field that had not been treated with insecticide, sifted, mixed with 20% sharp sand (in 2003), and sterilised at 120^{o} C for 20 minutes). The soil in the pot emergence traps was covered with moss to maintain humidity. Between 5 - 20 and 1 - 5 larvae of the same species were put in each pot emergence trap and corked tube, respectively. Larvae were placed on the soil surface and were allowed to bury themselves in the soil. The pot emergence traps and corked tubes containing the pest larvae were then left in the insectary on a shelf out of direct sunlight. The pot emergence traps were checked weekly and mist-sprayed with tap water if they appeared dry. Such treatment was unnecessary with the corked tubes, as the cork maintained the moisture conditions within the tubes.

Parasitoid collection

Once the first adult pests had emerged and were captured in the inverted plastic containers of the pot emergence traps or were seen inside the corked tubes, the soil was emptied into a white plastic tray placed under a magnifying glass and was carefully sorted using a fine paint brush and forceps. Any parasitoid cocoons found were collected. The cocoons (Figure 3) were put into a corked tube without any soil and left in the insectary for one month to allow the parasitoids to fully develop. The cocoons were then dissected under a binocular microscope (x6) and pre-emergence parasitoids (Figure 4) were removed and transferred into 100% ethanol for storage at 5°C before later identification.

Parasitoid identification

The female parasitoids that emerged from *P. chrysocephala* and *C. pallidactylus* larvae were identified using keys by Vidal, (2003) and Ferguson's 'A quick key to the identification of

Tersilochinae parasitic on insect pests of oilseed rape' (A. W. Ferguson, unpublished). Two morphological characters described in the keys were primarily used to separate the species of larval endoparasitoids of *P. chrysocephala* and *C. pallidactylus* belonging to Tersilochinae (Hymenoptera: Ichneumonidae): the length of sternaulus (a curved furrow dividing the lower part of the mesopleuron), and the proportion of the ovipositor sheath length to the length of petiolar tergite (tergite of first metasomal segment) (Figure 5).

Figure 1 Pot emergence trap

Figure 2 Corked tubes

Figure 3 *Tersilochus obscurator* cocoon Background grid is 1 mm

Figure 4 Pre-emergence *Tersilochus obscurator* ♀ removed from its coccon Background grid is 1 mm

Results

Rearing and identification of parasitoids of* Psylliodes chrysocephala *larvae

In 2002, between 29 April and 7 July, a total of 465 mature *P. chrysocephala* larvae were collected after their emergence from infested plant stems; 348 were reared in pot emergence traps and 117 in corked tubes (Table 1). Overall, 60 (13%) adult beetles emerged during summer; 10.9% in the pot emergence traps and 18.8% in corked tubes (Table 1). The first beetle emerged in a corked tube on 20 June; 23 days after placing the larva onto the sterilised soil. After sorting the soil of the pot emergence traps and corked tubes, 23 parasitoid cocoons were found (5% of total; 15 in the pot emergence traps and 8 in corked tubes) (Table 1). Two of these were put in corked tubes containing sterilised soil, and left in the insectary to emerge naturally. The remaining 21 cocoons were dissected and 21 (15♀, 6♂) adult pre-emergence larval parasitoids of *P. chrysocephala* were removed. An adult male parasitoid emerged on 24 March 2003 from one of the parasitoid cocoons left in the insectary. The other did not emerge.

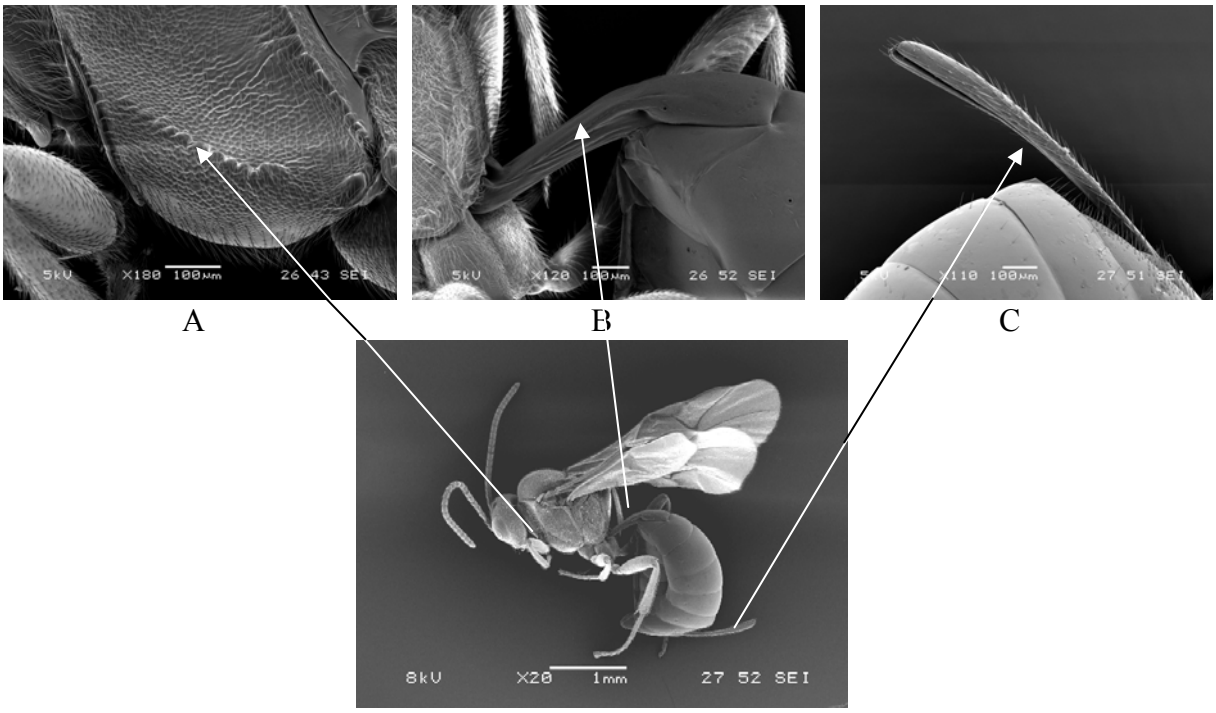

Figure 5 Scanning electron micrograph of morphological characters of *Tersilochus microgaster*, which are used to separate this species from *T. obscurator*
A) Sternaulus, B) Petiole, C) Ovipositor sheaths

Overall, mortality was 82.2%; 84.8% and 74.4% in the pot emergence traps and corked tubes, respectively (Table 1).

In 2003, 2991 *P. chrysocephala* larvae were collected between 23 April and 18 June (Table 1). In total, 1861 (62%) adult beetles emerged during summer; 28.7% in pot emergence traps and 68.1% in corked tubes (Table 1). The first adult beetle emerged from a corked tube on 13 June; 45 days after placing the larva on the soil. Thereafter, 383 parasitoid cocoons were found in the soil (12.8% of total; 21 in the pot emergence traps and 362 in corked tubes). All these were dissected and 383 (191♀, 192♂) adult pre-emergence larval parasitoids of *P. chrysocephala* were removed (Table 1). *Psylliodes chrysocephala* larval mortality in pot emergence traps and corked tubes was 66.6% and 17.7%, respectively (Table1); total mortality was 25%.

The 206 female adult parasitoids of *P. chrysocephala* larvae collected from the rearing procedures in both years were all identified as *Tersilochus microgaster* (Szépligeti) (Hymenoptera: Ichneumonidae: Tersilochinae). The identity was confirmed by comparison with voucher specimens held at Rothamsted Research by A. W. Ferguson.

Rearing and identification of parasitoids of* Ceutorhynchus pallidactylus *larvae

In 2002, from 31 May until 29 July, a total of 192 *C. pallidactylus* larvae were collected after they dropped from the damaged plants, and 144 were reared in pot emergence traps and 48 in corked tubes (Table 1). Overall, 30 (15.6%) adult beetles emerged during summer; 15.3% in the pot emergence traps and 16.7% in corked tubes (Table 1). The first beetle emerged in a corked tube on 4 July 2002; one month after the larva was placed onto soil. By sorting the soil of pot emergence traps and corked tubes, ten parasitoid cocoons were removed (5% of total; 7 in the pot emergence traps and 3 in corked tubes) (Table 1). Two of these were put in a corked tube containing sterilised soil, and left in the insectary to emerge naturally, and the remaining

eight cocoons were dissected. Eight (3♀ and 5♂) adult pre-emergence larval parasitoids of *C. pallidactylus* were removed (Table 1). Adult male parasitoids emerged naturally from both cocoons left in the soil in the insectary on 28 February 2003 and 5 March 2003. Larval mortality in pot emergence traps and corked tubes was 79.9% and 77.1%, respectively (Table 1); total mortality was 79.2%.

In 2003, from 14 June until 11 July, 102 *C. pallidactylus* larvae were collected (Table 1) and all were reared in corked tubes. Eight (7.8%) adult weevils emerged during summer. The first adult weevil emerged on 12 July; 28 days after placing the larva onto the soil. Thereafter, 10 (9.8%) parasitoid cocoons were found in the soil. These were dissected and 10 (6♀, 4♂) adult pre-emergence larval parasitoids of *C. pallidactylus* were removed (Table 1).

The nine female adult parasitoids of *C. pallidactylus* larvae collected from the rearing procedures in 2002 and 2003 were all identified as *Tersilochus obscurator* Aubert (Hymenoptera: Ichneumonidae: Tersilochinae). The identity was confirmed by comparison with voucher specimens held at Rothamsted Research by A. W. Ferguson.

Table 1 Number of pest larvae (*Psylliodes chrysocephala* [*P.chr*.] and *Ceutorhynchus pallidactylus* [*C.pall*.]) reared in the pot emergence trap (PET) and corked tube (CT) containers, the percentage of adult pests emerged and their parasitoids collected through rearing parasitoids in the insectary in 2002 and 2003

Pest	Year	No. pest larvae in soil		% adult pests emerged		% parasitoid cocoons found		% mortality		No. adult parasitoids reared	
		PET	CT	PET	CT	PET	CT	PET	CT	PET	CT
P.chr.	2002	348	117	10.9	18.8	4.3	6.8	84.8	74.4	3♂, 12♀	4♂, 3♀ *
	2003	446	2545	28.7	68.1	4.7	14.2	66.6	17.7	9♂, 12♀	183♂, 179♀
C.pall.	2002	144	48	15.3	16.7	4.9	6.2	79.9	77.1	4♂, 1♀	3♂, 2♀ **
	2003	–	102	–	7.8	–	9.8	–	82.3	–	6♂, 4♀

* Two parasitoids were withheld to emerge naturally; 1 male emerged, the other did not.
** Two parasitoids were withheld to emerge naturally; 2 Males emerged.

Discussion

Parasitoid rearing

Rearing the larval endoparasitoids of the stem-mining pests of oilseed rape, *P. chrysocephala* and *C. pallidactylus*, was more successful using the corked tube than the pot emergence trap containers. More parasitoid cocoons were found and mortality was lower in the corked tubes than in the pot emergence traps in both years of this study (Table 1). *Psylliodes chrysocephala* larval mortality was reduced from 82% in 2002 to 25% in 2003, and mortality in the corked tubes was 3.8 times lower than that in pot emergence traps (Table 1). These results suggest that corked tubes are more productive containers than pot emergence traps, possibly due to better water regulation properties of the former compared with the latter (see below). The number of *C. pallidactylus* larvae collected was low, but the percentage of parasitoid cocoons found was comparable to *P. chrysocephala*. Therefore, in future studies more plant samples need to be collected to evaluate the efficiency of this rearing method for *C. pallidactylus*.

Mortality of the pest larvae in the soil was high, particularly in 2002 (in total, 82.2% *P. chrysocephala* and 79.2% *C. pallidactylus*). According to our observations during sorting

through the soil, it seems there were at least two reasons for this mortality. The soil in many containers was too heavy and compacted and in some pot emergence traps was too wet. These conditions may have made the soil unsuitable for the larval/pupal development. These problems were resolved, to some extent, in 2003 by adding 20% sharp sand to the soil. This vastly improved success of rearing both adult pests and adult parasitoids from their larvae, particularly in the corked tubes.

Rearing parasitoid larvae to adult from mature host larvae has several advantages. In this study, the plant samples were collected at approximately 10-day intervals, and the pest larvae collected were mature and had left the host plant stems naturally. Such full-term larvae were more likely to be parasitized than those collected prematurely through plant dissection and extraction (Klingenberg and Ulber, 1994). The pest larvae were reared thereafter under natural environmental conditions (temperature and light regimes). Hence, the life history and phenological development of the pests and their parasitoids during our rearing procedures were more likely to be similar to that in the field than rearing in unnatural conditions (Shaw, 1997). During this investigation, both *P. chrysocephala* and *C. pallidactylus* third instar larvae were found to leave the plants and drop into the soil for pupation only during the night, because no pest larvae were observed in the drawers of cages during the day. The observed dates of emergence concur with previous studies; *P. chrysocephala* adults started to emerge in mid June (13-20) and *C. pallidactylus* adults emerged in early July (4-12). In field conditions, new generation adults of *P. chrysocephala* and *C. pallidactylus* were collected in emergence traps from 27 June onwards (Ferguson *et al.*, 2003). Also, by allowing some of the parasitoids to emerge naturally from the cocoons, we found that adult *T. microgaster* emerged on 24 March: a timing supported by Nitzsche (1998, cited by Ulber & Williams (2003)). The adult *T. obscurator* emerged at the end of February and the beginning of March. However, according to Ulber (2003), this parasitoid colonised oilseed rape in April or May, when the early larval instars of cabbage stem weevil occur within petioles.

Parasitoid identification

This study has confirmed, for the first time, that *T. microgaster* and *T. obscurator* are larval endoparasitoids of *P. chrysocephala* and *C. pallidactylus* , respectively in the UK.

The occurrence of *T. microgaster* in water traps placed in oilseed rape fields was reported for first time in the UK by Barari et al., (submitted). Here we confirm that this species is an active parasitoid of *P. chrysocephala* larvae in the UK by rearing it from its host. This species is a solitary endoparasitoid and has one generation per year (Ulber and Williams, 2003). It has also been reared from *P. chrysocephala* larvae in Germany (Klingenberg and Ulber, 1994), but its distribution elsewhere is unknown.

Tersilochus obscurator was reared from *C. pallidactylus* larvae. Here this species is confirmed as an active parasitoid of *C. pallidactylus* larvae in the UK by rearing it from its host. It is a solitary endoparasitoid and the most widespread and abundant parasitoid of *C. pallidactylus* larvae in Europe; reported from France (Jourdheuil, 1960), Germany (Lehmann, 1965; Klingenberg and Ulber, 1994; Nissen, 1997; Nitzsche, 1998), former Czechoslovakia (Šedivy, 1983), and the UK, Hungary, Ireland, Sweden and the Ukraine (Horstmann, 1981).

We found that identification of some *Tersilochus* species using only their morphological characters to be problematic. According to the latest identification keys (Vidal, (2003) and A. W. Ferguson (unpublished)), *T. microgaster* is separated from *T. obscurator* mainly by differences in the proportion of the ovipositor sheath to the length of the petiolar tergite, and the percentage of mesopleuron length spanned by the length of the sternaulus. In *T. microgaster*, ovipositor sheaths are ≤1.4 x the length of petiolar tergite, and the sternaulus

is defined by a series of pits extending over 50-80% of the length of the mesopleuron. In *T. obscurator*, ovipositor sheaths are ≥1.4 x the length of the petiolar tergite, and the sternaulus is weakly defined by a series of pits and extending up to half of the length of the mesopleuron. However, in this study, some (3% n=162) adult *Tersilochus* reared from *P. chrysocephala* larvae had morphological measurements exceeding this range; the proportion of the ovipositor sheath to the petiolar tergite ranged between 1.40-1.48, and in some specimens the sternaulus spanned more than 80% of the mesopleuron length. Molecular techniques using DNA sequencing are therefore being developed to aid the identification and separation of these *Tersilochus* species.

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Integrated Protection in Oilseed Crops
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Incidence of larval parasitism of *Psylliodes chrysocephala* within oilseed rape crops in Germany

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Abstract: The parasitism of cabbage stem flea beetle, *Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae) was investigated in various oilseed rape crops in Northern Germany from 1999 to 2003. The predominant parasitoid attacking the larvae in spring was identified as *Tersilochus microgaster* (Szépligeti) (Hymenoptera: Ichneumonidae), a solitary univoltine koinobiont endo-parasitoid. The phenology of emergence and the immigration of adult *T. microgaster* into new oilseed crops was monitored by emergence traps, yellow water traps and Malaise traps. Peak emergence of overwintering adults was observed in March from fields grown with oilseed rape in the previous year. Female parasitoids colonized the new oilseed rape crops from March to May, indicating a high level of synchrony between immigration of parasitoids and the appearance of larval instars of *P. chryso-cephala* within plants.

To study the level of parasitism of *P. chrysocephala* by *T. microgaster* host larvae were sampled from petioles and stems of oilseed rape plants from March to May. Parasitism was determined by dissecting the larvae. To obtain the adult parasitoids, sub-samples of host larvae were reared to pupation and emerging adult parasitoids identified in the laboratory. Percent parasitism ranged between 24.6 % and 44.4 % over the study. Superparasitism occurred regularly, with up to nine encapsulated eggs or larvae of *T. microgaster* per individual host larva.

Parasitism of *P. chrysocephala* larvae by *Aneuclis melanaria* (Holmgren) (Hymenoptera: Ichneumonidae) in autumn was only observed in one year. Ectoparasitism of larvae by *Trichomalus lucidus* (Walker) (Hymenoptera: Pteromalidae) was found only on very few host specimens.

Key words: *Psylliodes chrysocephala,* parasitoids, oilseed rape, *Tersilochus microgaster, Aneuclis melanaria,*

Introduction

The cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) is one of the most destructive pests of winter oilseed rape crops throughout northern and central European countries (Bonnemaison & Jourdheuil, 1954; Winfield, 1992; Alford et al., 2003). Adult females deposit the eggs close to the stem base into the soil from September until the following spring. Larvae feed within leaf petioles and in the developing stem, thereby destroying a portion of the pith and reducing plant vigour during winter. Feeding of larvae can start in September and goes on until the following April/May, however when the egg hatch is delayed by low temperatures in autumn, the occurrence of larvae may be restricted to the period of March to May/June (Nissen, 1997). Full-grown larvae leave the plants to pupate in soil.

Parasitoids have been found to provide substantial natural control of many important oilseed rape pests in Germany (Nissen, 1997; Nitzsche, 1998; Ulber, 2000). While the identity and status of the parasitoids of pollen beetle, seed weevil and pod midge has been studied extensively in the past, little information is available on the parasitism of cabbage stem flea beetle.

Materials and methods

Plant samples were collected repeatedly from oilseed rape crops at experimental sites near to Goettingen between November and May of the years 1999 – 2003. The larvae of *P. chrysocephala* were collected from petioles and stems and stored in 70% alcohol. The level of parasitism and superparasitism was determined by dissecting the larvae under a binocular microscope. The number of healthy and encapsulated parasitoid eggs and larvae was recorded. In order to rear out adult flea beetles and parasitoids from *P. chrysocephala* larvae in the laboratory, sub-samples of full-grown larvae were transferred to plastic boxes containing a 5 cm layer of sieved, moistened field soil (sandy loam). Adult flea beetles emerged within 2 – 3 weeks while adult parasitoids were extracted from the soil by wet sieving and identified after 8 – 10 weeks.

The phenology of emergence and immigration of adult *T. microgaster* into new oilseed crops was monitored in 2002 and 2003 by yellow water traps and on few occasions by emergence traps and Malaise traps. Eight yellow water traps were set up at ground level in a wheat crop which had been grown with oilseed rape in the previous year and in a new oilseed rape crop. Traps were half filled with water and a few drops of a detergent. They were emptied every Monday, Wednesday and Friday from February to May.

Results and discussion

Parasitoid species of P. chrysocephala *larvae*

In Germany, the larvae of *P. chrysocephala* have been reported to be parasitised by three species of ichneumonids, one species of braconid and one species of pteromalid (Ulber & Williams, 2003). In earlier studies conducted from 1953 to 1960 in Germany (Dosse, 1961; Lehmann, 1965), France (Aubert & Jourdheuil, 1958) and Czechoslowakia (Sedivý, 1983) the solitary univoltine endoparasitoid *Tersilochus tripartitus* (syn. *T. melanogaster* Thomson, *T. nigricans* Szépligeti) (Hymenoptera: Ichneumonidae) was found to be the most common species attacking the larvae of *P. chrysocephala* in spring. In France and Germany the parasitisation rates ranged from 30 to 60 % and from 3 to 27 %, respectively (Aubert & Jourdheuil, 1958; Dosse, 1961).

In our ongoing studies since 1990, *T. tripartitus* has never been detected; by contrast, *Tersilochus microgaster* (Szépligeti) (Hymenoptera: Ichneumonidae) was the most abundant and frequently occurring parasitoid of *P. chrysocephala* and designated for the first time to a specific host (Horstmann, 1971, 1981; Klingenberg & Ulber, 1994; Nitzsche, 1998). Being closely related to *T. tripartitus*, *T. microgaster* is a solitary koinobiontic endoparasitoid which parasitises the host larvae in spring.

The multivoltine larval endoparasitoid *Aneuclis melanaria* (Holmgren) (syn. A. diversus Szépligeti; *A. petiolaris* Szépligeti) (Hymenoptera: Ichneumonidae) was identified by rearing from the larvae of *P. chrysocephala* only in the autumn of 1999. The level of parasitism ranged between 1.9 and 5.2 % in various plots. No parasitism by *A. melanaria* was observed in autumn of 2000 and 2001, in spite of high levels of larval infestation and extensive dissections and larval rearing. In 2002 and 2003, the abundance of *P. chrysocephala* larvae in autumn was too small for further assessments.

Aneuclis melanaria has been reported to produce three generations per year (Aubert & Jourdheuil, 1958). Females parasitise the larvae of *P. chrysocephala* and *Ceutorhynchus pleurostigma* in autumn (Sedivy, 1983) while they attack the larvae of *Ceutorhynchus assimilis* and probably other hosts in June and July (Jourdheuil, 1960). In France, the parasitism of *P. chrysocephala* larvae by *A. melanaria* was very low, too, ranging between 0.2 % and 1.5 % in 1953, 1954 and 1955 (Jourdheuil, 1960).

In Germany the multivoltine braconid endoparasitoid *Diospilus morosus* Reinhardt (Hymenoptera: Braconidae) has been reported to parasitise the larvae of *P. chrysocephala* during autumn only rarely (Godan, 1950). In the present study from 1999 to 2001, parasitism by *D. morosus* was not found, even at very high host densities. Parasitism of cabbage stem flea beetle by *D. morosus* and *Diospilus oleraceus* Haliday has been reported from France (Jourdheuil, 1960); the level of parasitism proved to be very low. This has been attributed to insufficient synchrony of the autumn generation of *D. morosus* and the host larvae.

One female of the pteromalid larval ectoparasitoid *Trichomalus lucidus* (Walker) (Hymenoptera: Pteromalidae) (S. Vidal, pers. comm.) has been reared from a total of 260 larvae of *P. chrysocephala* which had been sampled from an oilseed rape crop at Goettingen in May 2003. This parasitoid has also been collected from two larvae in northern Germany (Nissen, 1997). In addition, in this study *T. lucidus* was found to parasitise the larvae of cabbage stem weevil, *Ceutorhynchus pallidactylus* (Marsh.).

***Phenology of* Tersilochus microgaster**

In wheat fields sown in the previous year to oilseed rape, peak emergence of overwintered adult *T. microgaster* was observed in 2002 and 2003 on 8 March and 28 March, respectively (Figure 1). In 2003, the last females occurred between 4 and 7 April. In the new rape crop the first individuals of *T. microgaster* were captured on nearly the same day as in the emergence field. In all years of study the females were trapped in oilseed rape crops from February/March up to the end of flowering in May, over approximately 9 weeks. A similar phenology of adult *T. microgaster* in oilseed rape crops was found at Goettingen in 1991, 1995 and 1997 (Klingenberg & Ulber, 1994; Nitzsche, 1998). In 1996 when the soil temperature remained below zero until the end of March, the first females occurred later, on 5 April. These phenologies have been confirmed by emergence and Malaise trapping.

The mean number of females per yellow water trap in both years was very small (2002: 8.1; 2003: 11.0) and was not clearly related to maximum daily temperatures. Malaise traps have been proved to capture female *T. microgaster* more effectively than yellow water traps (Nitzsche, 1998). The low effectiveness of yellow water traps as compared to suction traps has been also reported for many species of Tersilochinae by Sedivy (1983).

The differentiation between the males of *T. microgaster* and males of related species, e.g. *Tersilochus obscurator*, is extremely difficult (Horstmann, 19981). Therefore the number of male *T. microgaster* could not be analysed from trap samples.

***Percentage of larval parasitism by* T. microgaster**

This is the first report on the extent of parasitism of *P. chrysocephala* by *T. microgaster*. In 2001, 2002 and 2003, at peak levels of host abundance in the first ten-days of May, the field parasitism of *P. chrysocephala* amounted to 24.6 % (n = 280), 44.4 % (n = 792) and 22.8 % (n = 127), respectively. There was no positive relationship between the abundance of host larvae per plant and the level of parasitism. While in 2000/01 and 2001/02 high numbers of *P. chrysocephala* larvae were present within the oilseed rape plants throughout the winter, in 2002/03 the number of larvae started to increase only from the middle of March onwards. This might have affected the spatial-temporal coincidence between parasitoid and host populations resulting in different levels of parasitism.

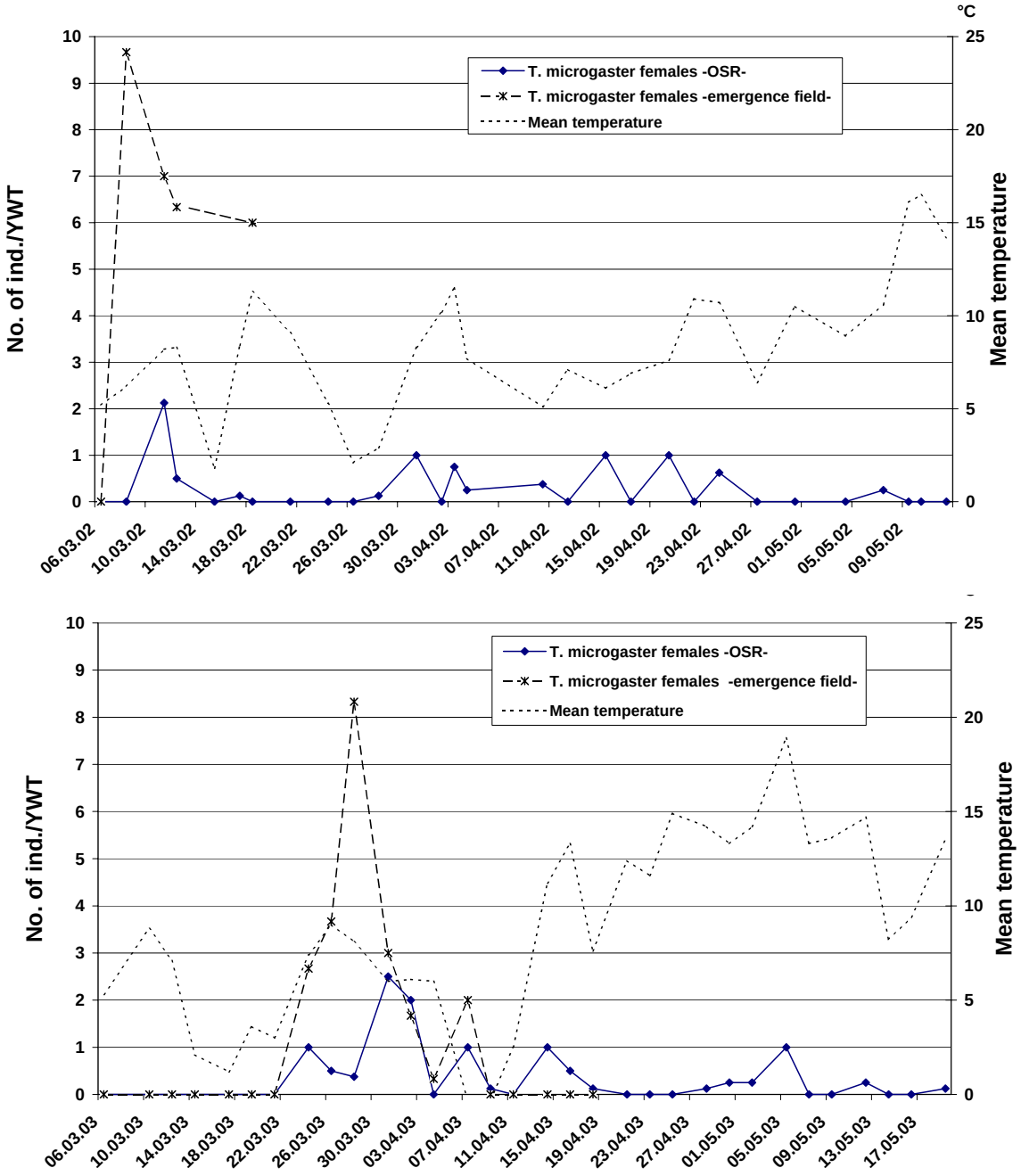

Figure 1. Number of female *Tersilochus microgaster* captured by yellow water traps (YWT) in a winter wheat crop (emergence field) and an oilseed rape crop (OSR) between March and May 2002 (top) and 2003 (bottom) at the experimental site in Goettingen, Germany.

In 2003, the temporal dynamics of parasitism was studied in greater detail (Table 1). Because the immigration of *T. microgaster* into the rape crop started on 21 March, no parasitism of larvae was found in samples collected on 17 March (Table 1). In plants sampled on 15 April, increasing numbers of host larvae were associated with 30.3 % of parasitism, indicating a high level of synchrony between the foraging activity of female *T. microgaster* and the appearance and accessibility of young larval instars of *P. chrysocephala* within petioles during the end of March and early April. Even if the abundance of host larvae continued to increase until 7 May, the percentage of parasitism remained high. The larvae which were developing late within the stems showed a lower level of parasitism.

Table 1. Effect of sampling date on the abundance of *P. chrysocephala* larvae and the level of parasitism and superparasitism by *T. microgaster* (Goettingen, Germany 2003)

Sampling date	Mean no. of host larvae/10 plants	% parasitism	% superparasitism
17 March	2.2	0	0
15 April	7.7	30.3	12.4
07 May	15.4	24.6	17.0
22 May	1.9	13.3	11.3

Superparasitism of *P. chrysocephala* occurred regularly, with up to nine encapsulated eggs and/or larvae of *T. microgaster* per individual host larva (Figure 2). The percentage of superparasitised host larvae varied only a little with time (Table 1). However, when superparasitism was related to the number of parasitised larvae as a standard of comparison, it increased from 41 % to 69 % and 83 % on 15 April, 7 May and 22 May, respectively.

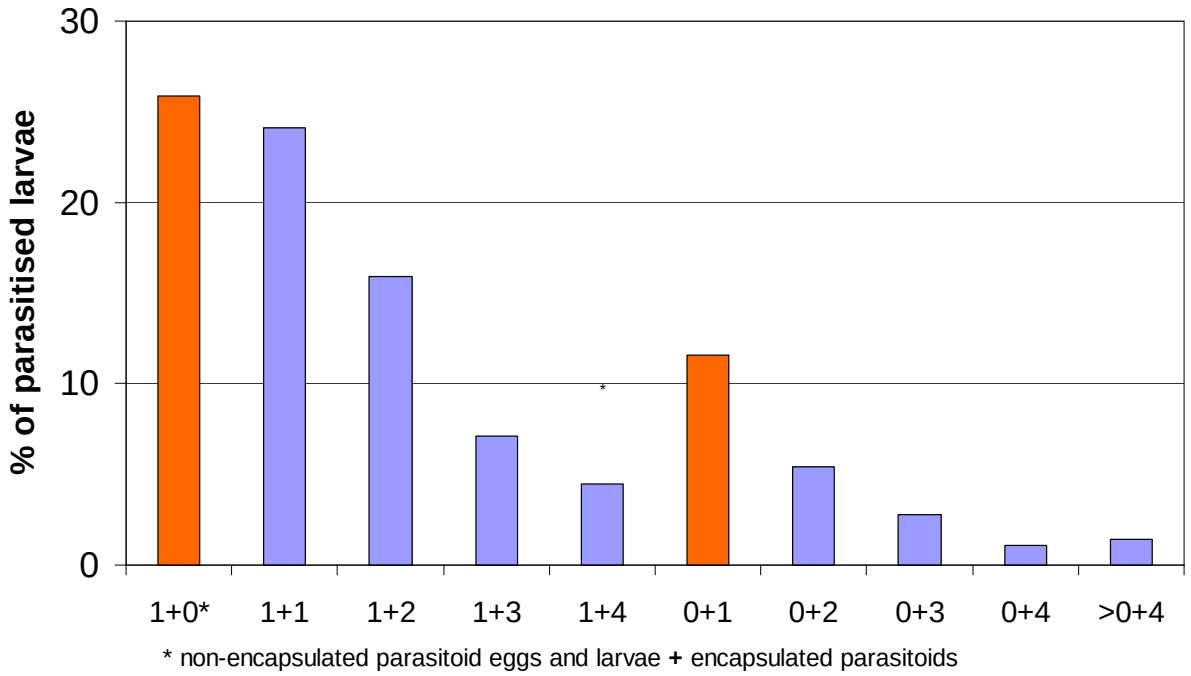

Figure 2. Superparasitism of *P. chrysocephala* by *T. microgaster* in oilseed rape plants sampled on 15 April 2002 at Goettingen, Germany. Total number of host larvae = 352.

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Predators

Integrated Protection in Oilseed Crops
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Incidence and feeding activity of epigeic, predatory invertebrates within winter oilseed rape in the UK with comparisons between integrated and conventional crop management.

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Abstract: The EU-funded project MASTER (MAnagement STrategies for European Rape pests) aims to realise the potential of predatory epigeic invertebrates in controlling six major pests of winter oilseed rape. This requires knowledge of the diversity of the predators and their diets. Pitfall trapping was used to assess the incidence and abundance of Carabidae, Staphylinidae and Araneae in integrated (ICM) and conventional (STN) oilseed rape plots. The activity density and diversity of all taxa was greater in the ICM plot. Gut dissections revealed that the larvae of *Meligethes aeneus* were eaten in the field by three species of carabid, of which the most abundant was *Nebria brevicollis*. There was also some evidence of predation on *Ceutorhynchus* sp. larvae and adults, although this was rare. The majority of Carabidae and all the Staphylinidae contained only liquid in their crops and Araneae are obligate liquid feeders; therefore a technique other than gut dissections is needed to further define the diet of these species in the field.

Key words: oilseed rape, integrated crop management, pests, predatory invertebrates,

Introduction

The MASTER (MAnagement STrategies for European Rape pests) project is a European collaboration researching integrated pest management strategies for oilseed rape (Williams, 2002; Williams *et al.*, 2002). One of the aims of MASTER is to enhance the activity of predatory invertebrates. The MASTER project targets six pest species, five of which are found in the UK: *Meligethes aeneus* (the pollen beetle), *Ceutorhynchus assimilis* (the cabbage seed weevil), *Ceutorhynchus pallidactylus* (the cabbage stem weevil), *Psylliodes chrysocephala* (the cabbage stem flea beetle) and *Dasineura brassicae* (the brassica pod midge). These are monitored in the crop, comparing their prevalence in an integrated crop management (ICM) plot and a standard European (STN) plot. The diversity and abundance of potential predators as well as their feeding activity must be elucidated before any attempt can be made to enhance their activity. The predators at the focus of this study are the insects and spiders that are active on the ground surface (epigeic), namely the Carabidae (carabid beetles), the Staphylinidae (rove beetles) and the Araneae (spiders).

Epigeic invertebrate predators have been widely recognised as important regulators of pest populations in arable crops (Krooss and Schaefer, 1998; Bohac, 1999; Kromp, 1999; Marc *et al.*, 1999). Much of the literature on their role as biocontrol agents focuses on those active in cereal crops with reference to aphid predation (Holland and Thomas, 1997; Collins *et al.*, 2002; Juen *et al.*, 2003). There is a dearth of literature on the feeding activity of epigeic predators in fields sown to oilseed rape.

There is an assemblage of carabids (about 30 species), staphylinids (about 30 species) and a few species of lycosid spiders that are associated with the regularly disturbed ground of the arable crop environment (Thiele, 1977); the latter can be very numerous during the summer. There is no reason to suppose that the diversity of the epigeic predatory fauna

associated with fields sown to oilseed rape is any different to that found in fields sown to other arable crops, although some differences may occur. For example, the carabid, *Amara similata*, has been shown to be more numerous in oilseed rape fields than in cereal fields (Luka *et al.*, 1998).

A recent study of the diversity, abundance and spatio-temporal distribution of carabids and oilseed rape pest larvae in a crop of winter rape in the UK (Warner, 2000; Warner *et al.*, 2000; Warner, 2001) showed ten species of carabid to be temporally associated with the larvae of MASTER target pests with a sub-set of these also exhibiting spatial co-incidence. The pests are most vulnerable to predation by epigeic predators during their egg and larval stages in the soil (*P. chrysocephala*) or when mature larvae drop from the plants to pupate in the soil (*M. aeneus, C. assimilis, C. pallidactylus, D. brassicae*). *Nebria brevicollis* was considered to be the most important potential carabid predator of *M. aeneus* larvae, *Agonum dorsale* of *C. assimilis* and *D. brassicae* larvae and *Pterostichus madidus* and *Trechus quadristriatus* of *P. chrysocephala* eggs and immature larvae (Warner, 2001; Warner *et al.*, 2003).

The feeding activity of this diverse fauna of epigeic invertebrate predators in oilseed rape fields, is almost unknown (Williams, 2004). There are numerous methods for elucidating the diet of predatory invertebrates ranging from gut dissections (Chiverton, 1988; Ingerson-Mahar, 2002) to state-of-the-art molecular techniques (see (Symondson, 2002) all of which have advantages and disadvantages.

The two aims of this study are as follows: 1) to determine the activity densities of Carabidae, Staphylinidae and Araneae in two systems of winter oilseed rape management (STN and ICM) and 2) to investigate, by way of gut dissections, the feeding activity of the epigeic invertebrate predators present in the two systems.

Materials and methods

Predator incidence

Transects of five pitfall traps were laid in each of two unreplicated, 1ha oilseed rape plots (STN and ICM) at Rothamsted Farm, Harpenden, Hertfordshire, UK. The traps were approximately 15m apart and consisted of a PVC sleeve into which a 150ml plastic beaker was inserted. A 4% sodium benzoate solution was used as a preservative with a few drops of nalgene detergent as a surfactant. The traps were opened on Mondays, beginning on 5 May 2003 and closed on Fridays each week until 4 July 2003. Trap catches were taken back to the laboratory where they were sorted and identified. Spiders were identified to family, carabids were identified to species and staphylinids were identified to genus.

Predator feeding activity

The crop contents of 601 carabids and staphylinids captured in the pitfall traps were examined. Each beetle was immersed in 70% ethanol and, using two pairs of fine forceps, its head and pro-thorax was teased away from the rest of body. This usually resulted in the anterior sections of the gut, including the crop, coming away with the head (Figure 1). In cases where the head came away without the crop, the remainder of the beetle's body had to be teased apart to free the crop. Once the crop had been isolated its contents were revealed by tearing it open with the forceps. The contents were then examined for fragments of target pests using a dissecting microscope. The pitfall trap catches were examined in chronological order and those beetles that consistently contained liquid in their crop in the earlier samples were not dissected in the later samples.

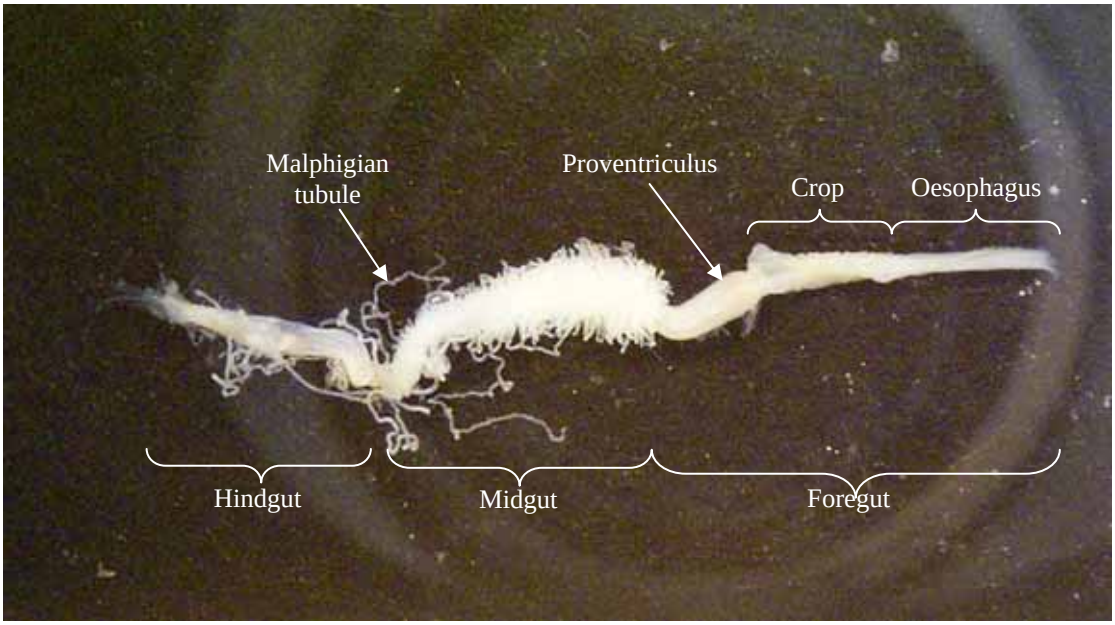

Figure 1. *Pterostichus madidus* gut. Entire except pharynx and posterior section of rectum. Note the empty crop; the villi covered midgut and several pairs of malphigian tubules branching from the midgut/hindgut junction.

Results and discussion

Predator incidence

Activity density of the trapped taxa was greater in the ICM plot than in the STN plot (Tables 1, 2 and 3). The activity density of the araneae, carabidae and staphylinidae were 8.3, 2.9 and 1.5 times higher, respectively, in the ICM traps than the STN traps. The differences within the activity density of the spiders between the two systems can be attributed to the lycosids and linyphiids (Table 1). The 'other spiders' were represented, mostly, by the tetragnathid, *Pachygnatha degeeri* and thomisids.

Seventeen and 15 carabid species were trapped in the ICM and STN systems, respectively (Table 2). The activity density of the three most commonly encountered carabids; *A. similata*, *N. brevicollis*, and *P. melanarius* were 5.4, 7.8, and 2.2 greater, respectively, in the ICM system than the STN system. The only carabids with a markedly higher STN activity density were *Asaphidion flavipes* and *Loricera pilicornis*. However, these species were not commonly encountered. Most of the Staphylinidae were identified to genus and the activity density of all the trapped genera was greater in the ICM plot, with the genus *Philonthus* being dominant (Table 3). Staphylinidae, represented by the grouping 'Other Staphylinidae' was an assemblage of several genera. The activity density of this grouping was greater in the STN plot than the ICM plot.

The differences in the incidences of the epigeic invertebrate predators between the ICM and STN systems were marked. The reasons for these differences are manifold. Firstly, the ICM system retains a layer of stubble and vegetable debris from the previous year's crop. This organic layer will provide habitats for eggs and immatures that are relatively safer, warmer and more moist than the comparatively stark environment of the STN system. The organic detritus also results in a more homogenous surface environment. This greater niche availability will support a larger and more diverse epigeic, predatory invertebrate fauna than

the relatively bare earth of the STN system. Other studies have shown that the presence of detritus on the ground can increase the number of potential prey species with a concomitant rise in predator numbers (Halaj and Wise, 2002; Agusti *et al.*, 2003)

Another important consideration is the difference in tillage between the two treatments. The ICM system is only minimally tilled whereas the STN system is ploughed. Many epigeic invertebrate predators will over-winter or spend their immature stages in the upper layers of the soil and are harmed by deep cultivation. It has been shown that no tillage or reduced tillage has a positive effect on the populations of pests and beneficial invertebrates (Horne and Edward, 1998; Cividanes, 2002)

Thirdly, insecticides were not applied to the ICM plot and although those applied to the STN plot were relatively selective some predators were probably killed by these applications.

Table 1. The incidence of Araneae (spiders) and their activity density in ICM and STN winter oilseed rape plots.

	Araneae			
Plot	**Linyphiidae (*Erigone*) sp.**	**Lycosidae**	**Other Spiders**	**∑**
STN	43	14	23	80
ICM	413	233	18	664

Predator feeding activity

Of all the potentially predatory arthropods sampled from the MASTER ICM and STN plots only three species of carabid were found to contain the remains of MASTER target pests in the crop: *N. brevicollis*, *P. madidus* and *P. cupreus*. No spiders were dissected as they are all, by nature, liquid feeders and would, therefore, contain no identifiable fragments in their crop. Extra cellular digestion is the common, if not sole, feeding strategy of the staphylinids, therefore, the crops of all the staphylinid individuals that were dissected contained liquids and no identifiable prey fragments. Many of the carabids were also found to have nothing more than fluids in their crops. The former species was most commonly found with MASTER target pest fragments in its gut.

By far the most common remains found in the guts of carabids were those of *M. aeneus* larvae. These were only found in those carabids trapped in the ICM plot (Table 4). 33.3% of the ICM trapped *P. madidus* were found to contain the remains of *M. aeneus* larvae (Table 4). However, *P. madidus* was only caught in very small numbers. Figure 2 shows the numbers of *N. brevicollis* with *M. aeneus* larval remains in their crops along with the numbers of *M. aeneus* larvae found to be dropping to the ground from the crop canopy for pupation. The incidence of *M. aeneus* larval drop was at its peak on 26 May with an associated peak in the incidence of *M. aeneus* larvae within the crops of *N. brevicollis*. (Figure 2). *N. brevicollis* was the most common carabid at the time of peak *M. aeneus* larval drop; therefore, this species of ground beetle may be an important predator of this pest species. Further work is needed to elucidate the density of this carabid at the time of peak larval drop. Other studies have shown that the density of carabids within an annual crop range from 1 to 96 per m^2 (Lovei and Sunderland, 1996). Densities between 14.5 and 1113 have been recorded from field boundaries (Lovei and Sunderland, 1996).

Table 2. The incidence of Carabidae (ground beetles) and their activity density in ICM and STN winter oilseed rape plots.

Coleoptera; Carabidae																			
Plot	***Amara similata***	***Nebria brevicollis***	***Pterostichus melanarius***	***Harpalus ruffipes***	***Pterostichus cupreus***	***Clivinia fossor***	***Amara plebja***	***Bembidion lampros***	***Pterostichus madidus***	***Asaphidion flavipes***	***Notiophilus biguttatus***	***Loricera pilicornis***	***Leistus spinibarbis***	***Harpalus affinis***	***Agonu dorsale***	***Demetrias atricaphilus***	***Trechus quadristriatus***	**Carabid larvae**	Σ
STN	35	23	34	2	2	0	3	2	14	16	9	16	6	1	5	2	0	0	275
ICM	189	181	76	27	44	25	21	12	12	10	8	7	6	7	4	2	1	7	805

Table 3. The incidence of Staphylinidae (rove beetles) and their activity density in ICM and STN winter oilseed rape plots.

Coleoptera; Staphylinidae								
Plot	***Philonthus* sp**	***Tachinus* sp.**	***Tachyporus* sp.**	***Xantholinus* sp.**	***Aleochara* sp.**	**Other Staphylinidae**	**Staphylinid larvae**	Σ
STN	22	9	15	5	5	48	1	105
ICM	76	28	24	5	0	26	7	166

The identifiable remains of *M. aeneus* larvae were also found in *P. cupreus* and *P. madidus*, however, these observations were rare. Fragments of *Ceutorhynchus* sp. larvae and adults were also found in the dissected carabid species, noted above, but their occurrence was, again, rare.

Table 4. Gut dissections of three carabid species and the incidence of *M. aeneus* larval remains within each.

	No. dissected (% with *M. aeneus* larvae in their crop)		
	Nebria brevicollis	***Pterostichus madidus***	***Pterostichus cupreus***
STN	22 (0)	15 (0)	2 (0)
ICM	163 (27)	12 (33.3)	33 (6.1)

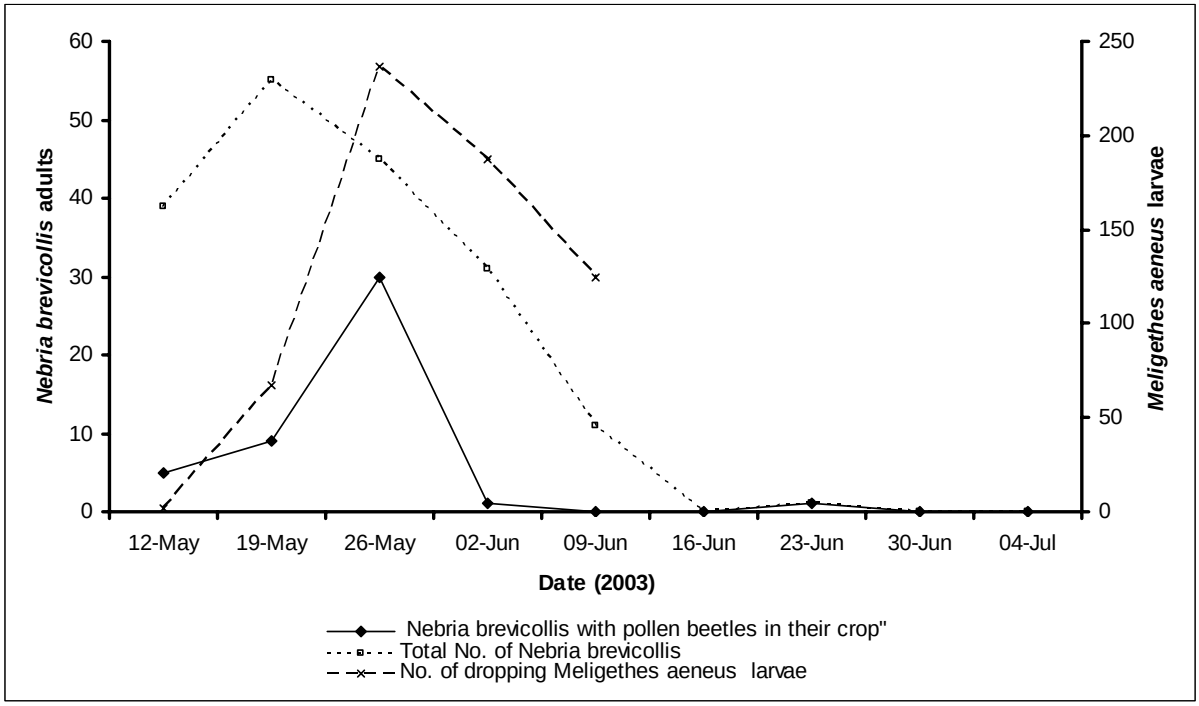

Figure 2. *Meligethes aeneus* larval predation by the carabid: *Nebria brevicollis* and numbers of *M. aeneus* larvae dropping from the crop canopy (per m^2).

Although, via gut dissections, MASTER target pests were positively identified from only three carabid species, it is very likely that a whole suite of predatory arthropods, present in oilseed rape fields, will feed on MASTER target pest larvae. The target pest species were found within the crops of three carabid species trapped in the ICM plot. No MASTER target pests were found in those carabids trapped within the STN plot. During the spring/summer insecticide was applied to the STN plot on the 8 April 2003 and 27 May 2003. These applications would have drastically reduced pest numbers. The carabids that survived these applications would not have the glut of dropping larvae available to those beetles in the ICM plot. Observations of *Ceutorhynchus* larvae in carabid crops was rare, but, again only carabids from the ICM plot were found to contain remains of these weevils. Plant dissections during the summer showed that *Ceutorhynuchus* sp. larvae were relatively common in the ICM, compared to the STN plot (R. Piper unpublished data). The dissected *N. brevicollis* trapped in the STN plot were found to contain more starchy material in their crops than individuals trapped in the ICM plot. *N. brevicollis* has been shown to be very catholic in its tastes (Davies, 1959; Sunderland, 1975) and this observation suggests that due to the dearth of other insect prey they resort to a more plant-based diet.

Other work has shown there are ten species of carabid that show coincidental distributions in time and sometimes space with all of the MASTER target pests apart from *C. pallidactylus* larvae (Warner, 2001). All of these species fed on MASTER target pest eggs or larvae to varying extents (Warner, 2001). However, carabids in a state of hunger in a laboratory situation will not behave the same as they do in the field. Of the ten important carabids mentioned by Warner (2001) *Loricera pilicornis* is an obligate predator of collembola (Bauer, 1982a), whilst *A. similata* is considered to be spermatophagous (Thiele, 1977). The large eyes and fast running, diurnal nature of *A. flavipes* also implies a reliance on collembola for food as these characteristics are shared with *Notiophilus biguttatus*, another obligate collembola feeder (Thiele, 1977; Bauer, 1982b). When these facts are taken into

account there are probably no more than five species of carabid that are important predators of MASTER target pests.

Gut dissections cannot tell us a great deal about the diet of those predatory arthropods that have liquid-filled crops, but it can provide unequivocal data on the diet of predatory species that contain identifiable prey fragments in their crop, something that state-of-the-art techniques, such as PCR amplification of prey DNA cannot do. However, PCR-based approaches have given insights into the diets of epigeic invertebrates in arable ecosystems that would have been almost impossible to elucidate using any other technique (Zaidi *et al.*, 1999; Symondson, 2002; Agusti *et al.*, 2003).

PCR-based approaches are valuable for studying predator-prey interactions and it is hoped that gut dissections will be used in conjunction with a PCR procedure to gain a more complete picture of the interactions that take place between MASTER target pests and their predators.

Acknowledgements

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Approaches to assess the importance of carnivorous beetles as predators of oilseed rape pests

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Abstract. The feeding ecology and efficacy of common carnivorous beetles from the families Carabidae and Staphylinidae as predators of the most important oilseed rape pest species are currently investigated within the EU-project "MASTER" (MAnagement STrategies for European Rape Pests). Using several experimental approaches, significant differences in feeding acceptance, food preferences and mean consumption rates, as well as in the degree of partial granivory of key predator species which proved to be dominant on winter oilseed rape fields in Braunschweig, Germany (Carabidae: *Amara similata*, *Anchomenus dorsalis*, *Poecilus cupreus*, *Pseudoophonus rufipes* and *Pterostichus melanarius*; Staphylinidae: *Tachyporus hypnorum*) have been revealed by our laboratory research so far. Selected results of laboratory feeding trials (choice and no-choice tests) are presented. In light of these findings, a comparison between the different beetle species according to their efficacy as oilseed rape pest predators seems possible.

Key words: oilseed rape pests, predators, Carabidae, Staphylinidae, consumption rates, preferences.

Introduction

Feeding trials with polyphagous predators conducted in the laboratory are contentious, as their results might not always reflect the natural feeding habits and flexibility in food choice of the predators - and of course they can hardly ever mirror the ecological heterogeneity seen in the field (Symondson 2002, McKemey et al. 2003). In spite of these limitations, such laboratory approaches can, nevertheless, provide helpful hints to certain, but hitherto unknown feeding preferences and reveal obvious differences between polyphagous predators like carabid species found sympatrically in agricultural ecosystems (Bilde & Toft 1997; Goldschmidt & Toft 1997; Suenaga & Hamamura 1998; Toft & Bilde 2002). Moreover, preference studies are difficult to perform in the field (Symondson 2002). The present study was undertaken as part of the EU-project MASTER (MAnagement STrategies for European Rape Pests), treating the task to investigate the feeding ecology of the most abundant key predator beetle species in oilseed rape fields, focussing on their consumption capacities and feeding preferences for the main rape pest species. To date, larvae of the pollen beetle *(Meligethes aeneus*), the cabagge stem weevil (*Ceutorhynchus pallidactylus*) and the brassica pod midge (*Dasineura brassicae*) have been studied. Basic laboratory feeding trials are supplimented by diagnostic techniques which analyze the predators digestive system (gut dissection and biochemical proof of the prey's DNA by Polymerase Chain Reaction). If the results of these different techniques and analyses are jointly interpretated, reliable and comparable assessments of the carabid and staphylinid key species influence on the main pest species and a supportable ranking of their impact seem possible. The present survey summarizes results of several laboratory feeding trials using carabid and staphylinid key predator species collected in an winter oilseed rape field near to Braunschweig, Germany.

Materials and methods

Larvae of the oilseed rape pest species *M. aeneus*, *C. pallidactylus* and *D. brassicae* were collected in the field by funnel traps and by beating directly from the flower heads, by plant stem dissection or from sieve buckets filled with infected oilseed rape pods, respectively. Predators were collected in pitfall traps and maintained in permanent laboratory cultures. Feeding trials were performed in small round plastic dishes (10 cm diameter, 3.5 cm in height) in a laboratory climate chamber at 20 C, 80 % r.h. and long-daylight conditions. The predator specimens were seperated and placed individually in the dishes. A small, flat plastic plate (25 cm diameter) for offering the prey larvae was also placed inside each dish. Before starting the experiments, each beetle was starved for 48 hours. A no-choice-feeding trial was performed with larvae of the pod midge *D. brassicae* as prey. The prey was killed by freezing prior to experiments. Larvae of *D. brassicae* were offered daily to each beetle in carefully chosen amounts (5, 10 or 15). Five carabid species that appeared to be the most dominant predators in the oilseed rape field: *Amara similata*, *Anchomenus dorsalis*, *Poecilus cupreus*, *Pseudoophonus rufipes* and *Pterostichus melanarius*, were tested in the experiment. In addition, a choice test with two facultative granivorous species, *A. similata* and *P. rufipes* and the predominantly carnivorous *P. cupreus* was performed to examine differences in their food composition. Loose oilseed rape seeds and larvae of *D. brassicae* were given simultaneously each day to individual test beetles. Another experiment was performed using only specimens of *A. similata,* offering a larva of *C. pallidactylus* together with an oilseed rape seed each day. A further choice-test was conducted using the staphylinid *T. hypnorum* and *M. aeneus* larvae as prey, because this staphylinid species is a suspected substantial predator of pollen beetle larvae. One larva each of *M. aeneus* and *D. brassicae* were given to the predator for 24 hours.

Results and discussion

In the no-choice test, it was evident that most of the key predator species varied significantly in their mean daily consumption rates of *D. brassicae*-larvae (Figure 1). This was not always merely due to differences in their body size and physical feeding capacities. For instance, *P. rufipes*, a species which has a much bigger body size than *A. similata*, showed a significantly lower daily feeding rate than the latter. Also, the mean daily consumption rate of *A. similata* was as high as that of *P. cupreus*, in spite of the reputation of the former as a mainly granivorous or phytophagous species (Jorgensen & Toft 1997b, Büchs 2003).

In the choice-test offering oilseed rape seeds and larvae of *D. brassicae*, significantly different degrees of entomophagy between *P. rufipes* and *A. similata* were revealed. *P. rufipes* showed a significantly higher daily consumption of rape seeds, while *A. similata*, in spite of its smaller size, consumed significantly more pod midge larvae per 24 hours. The relationship between carnivory and granivory of *A. similata* thus resembles that of *P. cupreus*. In light of our study, the feeding behaviour of *A. similata* as being mainly phytophagous or granivorous may need to be reconsidered, for instance when compared to *P. rufipes* which has a similar reputation (Jorgensen & Toft 1997a; Honek et al. 2003) and exhibits a significantly more balanced feeding ratio between larvae and seeds. Therefore, *A. similata* reveals a much higher degree of carnivory than previously reported (Jorgensen & Toft 1997b; Luka et al. 1998). This hypothesis is also confirmed by the trial which provided a choice between a rape seed and a larva of *C. pallidactylus*. In 36 cases, only the larva of *C. pallidactylus* was consumed, in 12 cases both food items were taken, and in no case only the rape seed. Therefore, *A. similata* significantly preferred the weevil larva ($p<0,01$; chi-square test; $\chi^2 = 12.0$;

N = 48). The assumed predatory importance of *A. similata*, which might be substantial because this carabid species is by far the most abundant in oilseed rape fields and shows the most expedient phenological coincidence with the period of oilseed pest larval dropping (Büchs 2003), is currently being examined in our further research

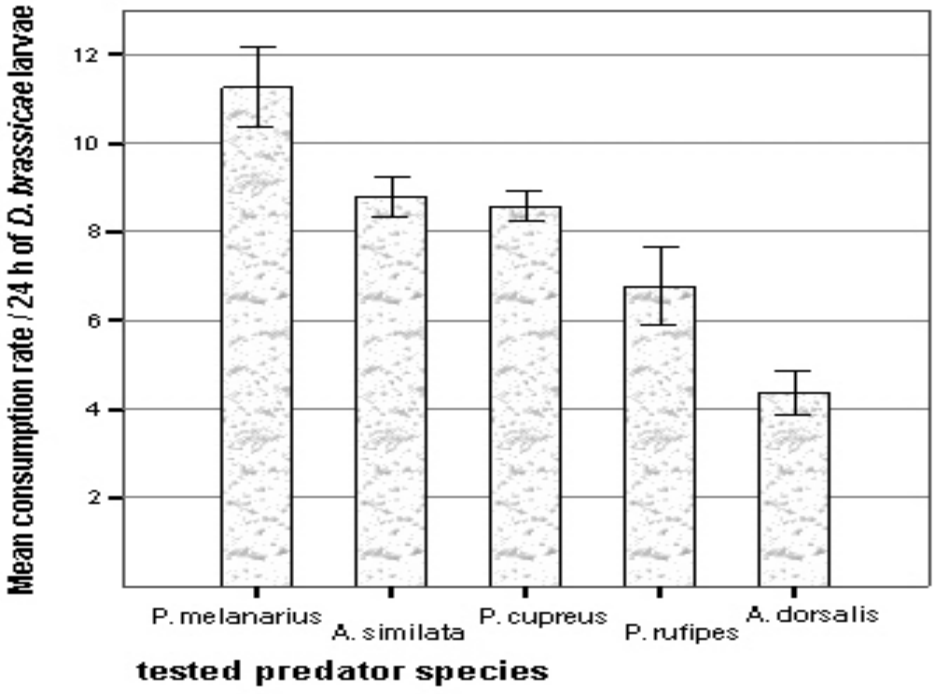

Figure 1. No-choice tests with 5 carabid species using larvae of *Dasineura brassicae* as prey; mean consumption rates per 24 hours (N = 760). All columns differ significantly when compared pairwise (*A. similata* - P. *rufipes*: $p<0.01$; other pairs: $p<0.001$; Mann-Whitney-U-Test) except for *A. similata* compared to *P. cupreus* ($p>0.5$).

Distinct and significant prey preferences for a certain oilseed rape pest species could not be found for the polyphagous carabid species in our choice tests. However, the staphylinid *T. hypnorum* preferred the larvae of *M. aeneus* highly significantly over the larvae of *D. brassicae* (29:5 cases; chi-square test; $\chi^2 = 16.9$, $p<0.001$;) and other offered food items, so an adaptation for this prey might be assumed and needs to be tested by further trials. By integration of other key predator species, new combinations of rape pest prey larvae and vegetable food items in choice trials and changes in the experimental design, the results presented here shall be confirmed and supported in a more complex experimental environment. After consolidating these feeding trials with results of microscopical and biochemical analyses of the predators gut contents, our aim will be to compile a provisional ranking list of the key predator species according to their potential as natural enemies of oilseed rape pest larvae. For sure, these experimental approaches can not be considered in isolation as a reliable assessment of the importance of the key predator species in oilseed rape fields. Aspects of spatio-temporal activities and their relation to prey population dynamics, population sizes of the different predacious species, and influences of inter- and intraspecific competition as well as the farming system have to be considered to accurately estimate the impact of a predator species on the different pest species in a complex agricultural system.

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Impact of predators on pollen beetle *Meligethes aeneus* on rapeseed in Finland

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Abstract: Predatory arthropods occurring in turnip rapeseed fields in southern Finland were examined in three separate studies in 1983-85, 1993, and 1999-2000. Only a few euconstant species were found in rapeseed in Finland; these include the carabid beetles *Pterostichus melanarius, Amara eurynota, Harpalus rufipes, Calathus melanocephalus*, and *Clivina fossor*. More than 40 species of carabid beetles were identified from rapeseed fields; more than half of all ground beetle species occurring in arable crops in the study area. A large guild of generalist predators occupies fields in Finland, where their activity densities vary greatly between crop types and years. In most field crops with low plant density (and diversity), e.g. cabbage, sugarbeet, and rapeseed, predator activity densities are at their lowest in Finland in July – at the time of pollen beetle *Meligethes aeneus* pupation. Consequently, the predator impact on pollen beetle during pupation was found negligible under current cropping practices in spring turnip rape in Finland. However, activity densities of predators, and pollen beetle numbers, may be amenable to manipulation e.g. via intercropping techniques.

Key words: activity density, agroecosystem, carabid beetles, conservation biological control, intercropping, ladybeetles, natural enemies, oilseed crops, pitfall trapping, predator impact, spiders, staphylinid beetles

Introduction

Polyphagous predators have been shown to be important natural enemies of pests of oilseed rape under certain conditions. In Germany, Thiele (1977) concluded that about 50% of pollen beetle *Meligethes aeneus* larvae/pupae may be eaten by predatory ground beetles during larval drop and pupation in soil. Many studies on polyphagous predators, in particular carabid beetles, in agricultural fields have been carried out in Finland, but none in rapeseed crops. Practically all rapedseed grown currently in Finland is spring turnip rape (*Brassica campestris*, about 60 000 ha), with a few 1000 ha of spring oilseed rape (*B. napus*) grown in southwestern part of the country. Winter rapeseed has been tried occasionally, but overwintering losses are usually too high.

As pollen beetle is the most important, and in most years, the only pest of significance in turnip rapeseed in Finland (Hokkanen, 2000), we have carried out studies on its natural enemies for over 20 years. Due to the different cropping system and crop phenology, the significance of predators as pollen beetle natural enemies could not be extrapolated from the results in other countries. Therefore the aim of our studies has been to (i) assess the predator fauna occurring in rapeseed fields in Finland, (ii) study their potential impact on pollen beetle numbers, (iii) evaluate the impacts of some agricultural practices (use of insecticides, intercropping) on the ability of predators to lower pollen beetle numbers.

Material and methods

Predators of the pollen beetle were studied in 1983-85, 1993, and 1999-2000. *The occurrence of generalist predators* in rapeseed fields was investigated mainly by pitfall trapping as follows:

1983:	27.6.- 9.8.	4 separate fields, 5 pitfall traps in each
1984:	28.6.-27.8.	2 separate fields, 5 traps in each
1993:	24.5.-23.8.	24 pitfall traps in one large field (2 treatments, 4 reps)
1999:	15.5.-31.8.	32 pitfall traps in one large field (4 treatments, 4 reps)
2000:	15.5.-31.8.	32 pitfall traps in one large field (4 treatments, 4 reps)

Additional data on predator occurrence were obtained with yellow water traps in 1983 and 1984, by sweep netting in 1984 and 1985 (including twice around the clock every 2 h for nocturnal predators); with insect glue on 15 rape plants in 1983 to monitor climbing predators; and by visual observations and direct counting in rapeseed fields annually 1984-1995 during larval sampling over all of southern Finland.

Impact of predators on pollen beetle numbers was investigated first with exclusion experiments in 1985. These employed 8 plots, 2 m x 2 m each, fenced to exclude epigeial predators, and 7 reference plots (i.e., total of 15 experimental plots). 15 photoeclectors (one per plot, each 1 m^2) were erected after pupation of pollen beetles, and the emerging pollen beetles were collected and counted (n = 11 105 individuals).

More data on predator impact were gathered as part of an investigation on the effect of crop plants and plant diversity on predation (intercropping experiments). Strip intercropping of rapeseed with red clover was experimented in 1992-1993; among the parameters measured were predator activity densities, pollen beetle colonisation, level of parasitism by parasitoids, and size of pollen beetle F1 generation.

Results

Carabid and staphylinid beetles along with spiders were the most common predators encountered. Over 40 species of ground beetles were determined, which is more than half of the number of carabid species reported from arable crops in the Viikki Experimental Farm (Varis & Holopainen 1987). There was a large variation between years and separate fields in the species composition (Table 1), with only a few species occurring predictably (euconstant species). These include *Pterostichus melanarius, Amara eurynota, Harpalus rufipes, Calathus melanocephalus,* and *Clivina fossor* (Table 1).

Besides ground beetles, other potentially important predatory groups in the rapeseed agroecosystem in Finland were encountered as follows:

Staphylinid beetles: very common in pitfall traps, yellow water traps, and in photoeclector catches. Abundance is roughly one-half of the numbers of carabid beetles.

Spiders: as Staphylinids.

Lacewings: encountered only occasionally, but regularly.

Predatory bugs: encountered only occasionally; *Nabis ferus* and *Anthocoris nemorum* were identified.

Predatory ladybeetles: Usually very low numbers on rapeseed. However, in the exceptional year of 1988, following a 'ladybeetle explosion', significant predators of pollen beetle

larvae on all rapeseed fields in southern Finland: typical densities were 40-50 ladybeetles / m^2 over all of the rapeseed fields (*Coccinella septempunctata*, *Adalia bipunctata*).

Table 1. Dominance percentages of carabid beetles in turnip rapeseed fields in Finland. Total catch was 8508 individuals from pitfall traps at the Helsinki Univ. Experimental farm in Viikki

	1983+1984	1993	1999+2000	Overall
Pterostichus spp.	45.7	9.5	45.7	33.6
P. melanarius	34.1	–	28.6	–
P. niger	9.6	–	5.6	–
Amara spp.	8.5	7.4	19.4	11.8
A. eurynota	–	–	16.4	–
A. bifrons	–	–	2.8	–
A. aulica	–	–	0.1	–
Harpalus spp.	3.8	10.1	18.5	10.8
H. rufipes			8.6	
H. rufibarbis			6.0	
H. affinis			3.3	
Calathus melanocephalus	2.5	5.1	15.3	7.6
Bembidion spp.	0.1	17.0	4.4	7.2
Clivina fossor	2.2	9.3	2.4	4.6
Trechus spp.	8.9	37.0	–	–
Lasiotrechus discus	–	16.8	–	–
Patrobus atrorufus	5.0	–	–	–
Agonum dorsale	0.1	4.0	–	–
Carabus granulatus	–	–	3.2	–
Carabus hortensis	–	–	1.8	–
Synuchus nivalis	–	2.7	0.1	–
Lebia sp.	–	–	0.4	–
Loricera sp.	–	–	0.2	–

Impact of insecticide treatment on predator activity was studied in two occasions: in 1983 fenitrothion was sprayed on one part of a field, and the other half was untreated. Spraying had a drastic effect on carabid activity: 3-fold more were captured on untreated part. Species that appeared particularly affected: *Lasiotrechus discus, Patrobus atrorufus,* and *Synuchus nivalis.*

In 1999-2000 lambda-cyhalothrin treatments were carried out in 10 m x 10 m plots (4 replicates). No differences were detected in carabid activity densities between untreated, insecticide treated, or *Metarhizium*-treated plots.

Impact of predators on pollen beetle numbers were studied in 1985 and in 1993.

(1) Exclusion experiments in 1985 showed that there was no significant impact on the number of emerging pollen beetles from predator exclusion (total of 11105 beetles were collected from the emergence traps). A likely explanation is the seasonal dynamics of carabids in annual crops in Finland: the activity is at its lowest during pollen beetle pupation,

2nd and 3rd week of July. Activity density dynamics differ, however, by the crop: data from rapeseed, sugarbeet, cabbage, and timothy indicate that in more permanent crops (e.g., timothy) some key carabid species maintain activity throughout the summer (Varis et al. 1984).

(2) Intercropping experiment in 1993 revealed no differences in the number of pollen beetles/plant, %-parasitism, or overall predator activity densities between the monocrop and intercrop. The number of emerging new generation pollen beetles, however, was drastically reduced in the intercrop as compared with the monocrop, which produced about 5 times as many F1 pollen beetles as the intercrop per surface area, or about 2.5 times as many per rapeseed plant (Fig. 1). Higher predator pressure (lower total number of prey, but equal number of predators) in the intercrop is a likely explanation, worth of further investigations.

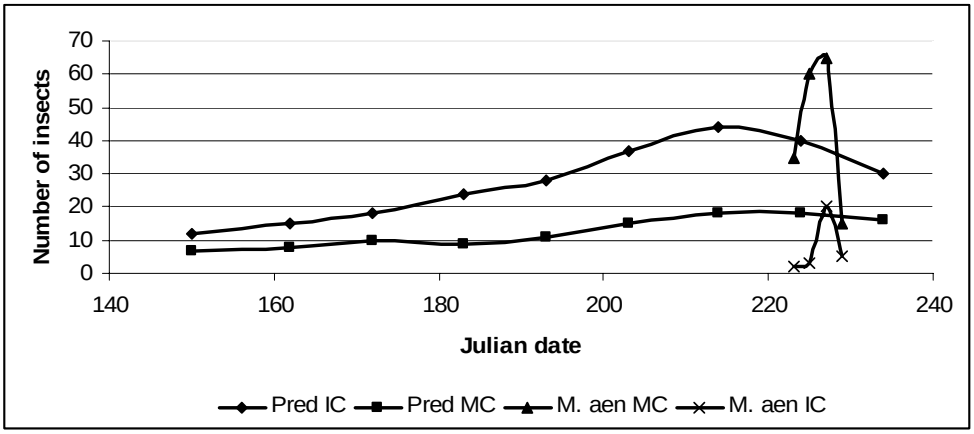

Fig. 1. Number of predators per pitfall trap, relative to rapeseed plant density, in the monocrop (MC) and intercrop (IC), and number of *M. aeneus* F1 / m^2 from both systems. Pupation occurs around day 200 in Finland.

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Long term survival of Brassica Pod Midge (*Dasineura brassicae*) populations

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Abstract: The brassica pod midge is a small fragile insect with a short life of only 1 or 2 days and with limited ability for long distance migration. It has developed a strategy to survive during unfavourable conditions that minimises the risk of extinction. Emergence over at least a 5 year period and during up to two month is described as parts of this strategy.

Key words: Brassica pod midge, *Dasineura brassicae*, long term survival, emergence

Introduction

Economic crop damage by Brassica Pod Midge (*Dasineura brassicae*) is quite rare in Sweden, both in winter and spring oilseed crops. Most of the damaged pods are concentrated in the first 30-60 meters from the crop edge, whereas the main part of the crop shows almost no damage (Sylvén 1949, Thiem 1970). In Sweden, no pesticides have been registered for use during flowering before 2000. The Swedish situation reflects a situation when natural biological control and environmental factors determine the damage levels

More severe damage is seen very seldom. However, in 1992, in the eastern part of central Sweden, winter rape was heavily attacked and more than half of the pods were lost in some crops. Table 1 show results from an annual survey. Crops were sampled along a tramline and 20 plants taken at random. Pods and pod midge damaged pods on the main raceme and the two next side branches were counted and mean % damaged pods per crop calculated.

Table 1. Mean pod midge damage in the county of Östergötland 1992 – 1997.

	1992	1993	1994	1995	1996	1997
Mean % damaged pods	30.6	2.8	7.7	7.0	6.7	1.4
SE	18.2	4.1	7.7	5.1	6.6	1.2
Range	6-60	0-10	0-31	2-18	1-21	0-5
Number of crops sampled	18	5	29	21	11	23

Even if there is a heavy attack, as in 1992, this does not influence the level of attack during subsequent years. The extent of damage in a year is determined by the conditions of that year. After a year with many attacked pods the populations of pod midge are much higher. This apparently does not increase the risk of a continued high damage level in subsequent years. The pod midge is almost totally dependent on a number of factors that will determine the reproductive success in a specific year.

(1) The midge lives for only a few days and weather during this period must allow migration to rape crops from hibernation fields. Oilseed rape crops must be located within a few kilometres from the emergence site (Schütte 1965, Sylvén 1970).

(2) In the rape crop, suitable pods must be present, e g emergence and migration must be synchronised with the rape plant development.
(3) Cabbage seed weevil (*Ceutorhynchus assimilis*) should be present. The female midge uses the feeding punctures of the seed weevil for oviposition (Speyer 1921, Sylvén & Svensson 1975).

Five factors: midge population, weevil population, crop development, crop location and weather conditions will only be favourable to the midges in a random manner. In some years, the potential for the midge to multiply will thus be very small and without special strategies for survival the populations could be extinguished. One survival strategy is the prolongation of the hibernation and a very long emergence period. This will be described in this paper.

Material and methods

Emergence of pod midge from fields that had had winter oilseed rape was followed on 18 farms during 1993 - 1997. Wooden emergence boxes were used, and the midges were caught in a glass tube inserted in one wall of the box and closed in the other end with a piece of nylon net. Three boxes were used in each field and they were emptied twice each week from mid May to early July.

For five of the farms, we followed emergence in the same field for five years following the rape year 1992. In other fields, we followed the emergence for 4 years, three years and so on; all investigations terminated in 1997. Here we present data from 6 farms and the emergence from those fields that had winter oilseed rape during 1992 – 1994. These fields were followed until 1997, e. g. for 5, 4 and 3 years.

Results and discussion

The pod midge has 2-4 generations each year. An increasing part of each generation hibernates, and a small part of them will stay in the soil for more than one year. In figure 1 four crops with many damaged pods in 1992 were monitored for 5 years. The emergence of midges from these breeding fields lasted for 5 years. In the beginning many midges emerged, and later fewer. Figure 2 and 3 show the emergence from other fields on the same farms. A high population level in the soil results in a much higher emergence in subsequent years. At lower population densities, the number of midges that emerged is much more unpredictable and soil conditions can be an important factor (Jönsson 1988). As an example, there was no emergence at all in many fields in 1995, both first and second year fields after the rape year. Several generations during the same season and the re-infestation of the plants by part of the first and to some extent also subsequent generations is also an adaption to increase synchronisation with the host plants. The midges that can successfully breed in the first generation will produce a more synchronised second generation. The “random” appearance of the midge will of course also affect the biology of the pod midge parasitoids. The most successful parasitoid, *Platygaster*, show the same pattern of several generations that hibernate partially (Buhl 1960).

The pod midge has a high capacity to multiply, once the conditions are favourable. A female can lay several batches of about 1-35 (mean 15) eggs (Åhman 1985). Emergence over a 5-year period from a single successful year ensures that there are midges, although in low numbers, even after years when breeding has failed totally.

The emergence period is also extended (Fig. 4.). Emergence of pod midges started in the middle of May (during early flowering of rape) and continued until the end of June or the beginning of July.

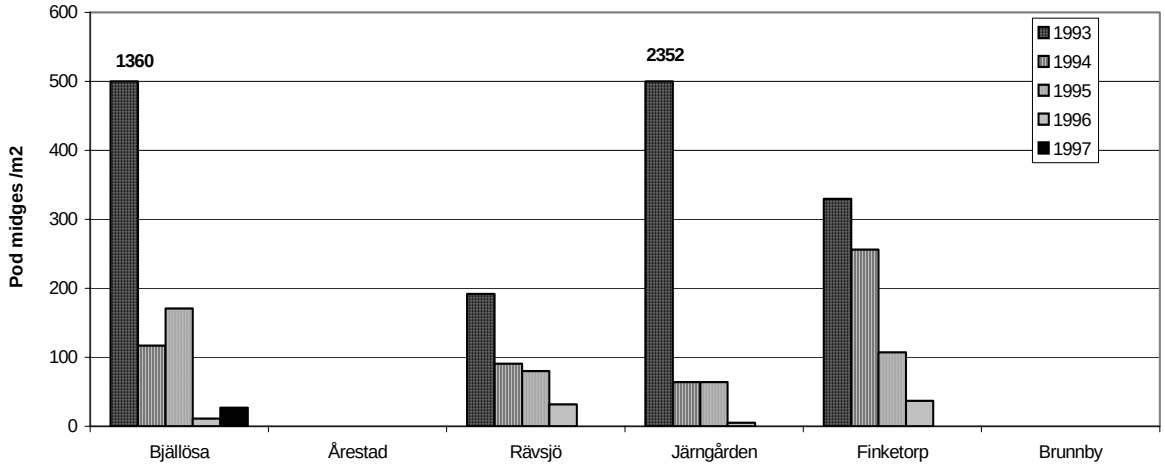

Figure 1. Emergence of brassica pod midge during 1993 to 1997, from four fields that had winter oilseed rape in 1992.

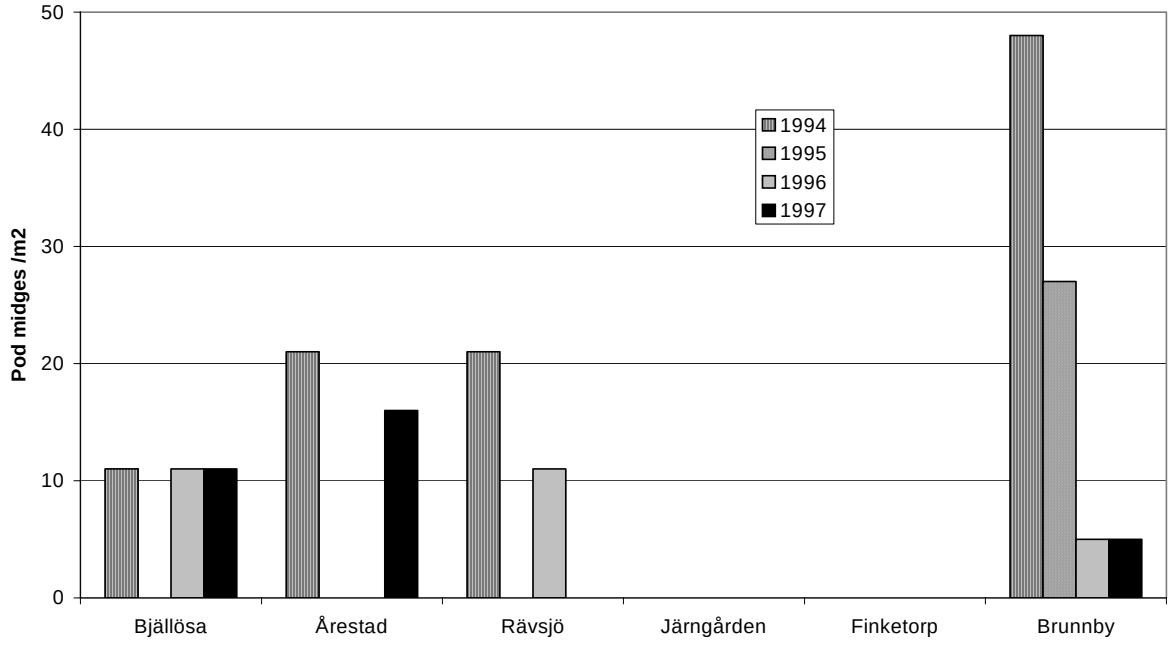

Figure 2. Emergence of brassica pod midges during 1994 to 1997, from four fields that had winter oilseed rape in 1993.

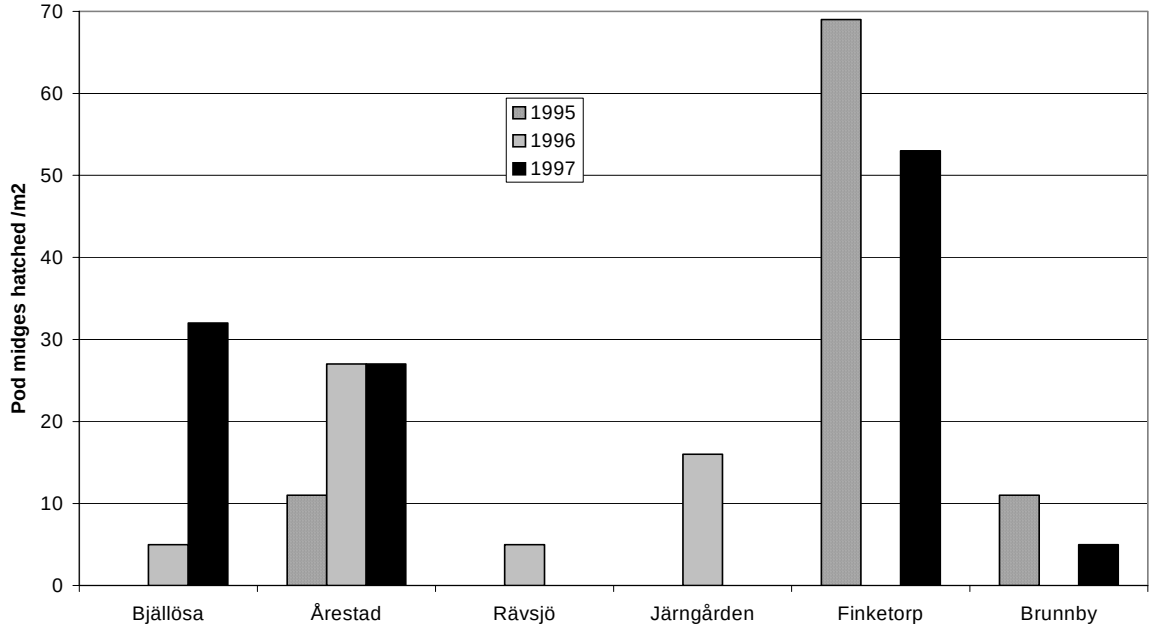

Figure 3. Emergence of brassica pod midges during 1995 to 1997, from six fields that had winter oilseed rape in 1994

Emergence will cover pod development stages of both winter and spring rape crops in a region. Emergence also takes place in the early morning (Sylvén 1970), which makes it possible for the midges to migrate during a period of the day when temperature and humidity are more favourable. During sunny summer days, wind speed is often building up during the day, and storm conditions with showers can be reached in the afternoon. Migration conditions should be safer in the first part of the day than later. The brassica pod midge is a small fragile insect with a short life, only 1 or 2 days (Sylvén 1949) and with limited ability for long distance migration. It has developed a strategy to survive during unfavourable conditions that minimises the risk of getting exterminated:

(1) Emergence can be extended over at least 5 years
(2) During years with unfavourable weather conditions, most probably very dry years, no emergence will occur.
(3) Emergence is extended over a two month period. Part of the population will always find suitable growth stages for oviposition, either in winter or spring brassica plants.
(4) Emergence takes place in the morning, when migration conditions more often are better that later during the day.
(5) If the cabbage seed weevil is absent, the female midge can still penetrate young pods with her ovipositor. This can only be done during a very short period of pod development (Stechmann & Schütte 1978) and will only permit the midge population to survive at a very low density.

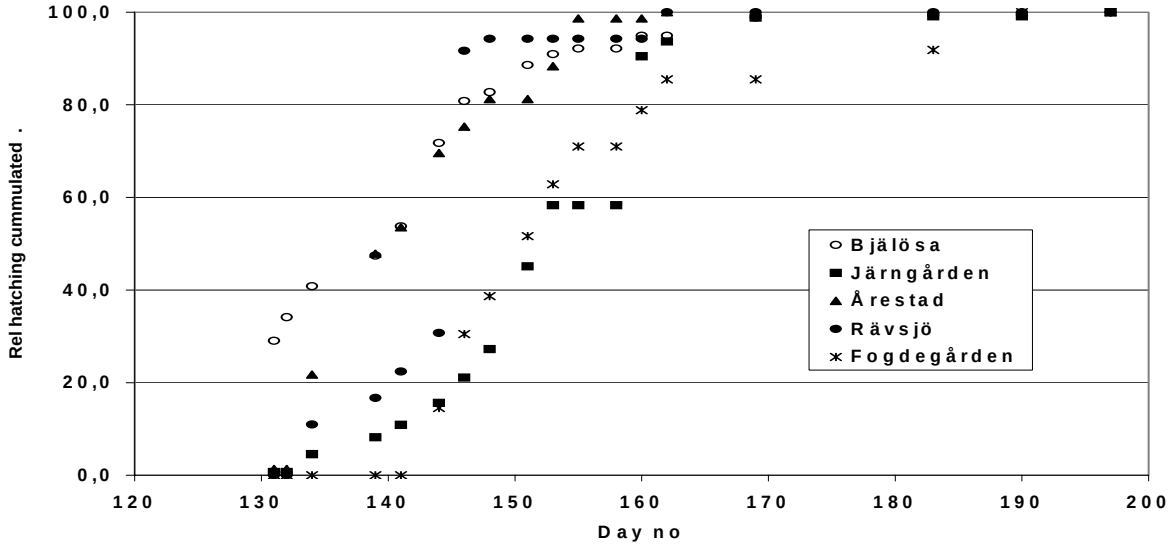

Figure 4. Relative (%) cumulated emergence in 1993 from fields with winter rape in 1992. Day no 130 is in the first week of May and 200 in the middle of July

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