Sustainable Arable Production Link (SAP11143)

"Novel Strategies for Aphid Control using Entomopathogenic Fungi"

Final Report

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Aims of Project

The overall objective of the project was to **t**o provide an understanding of the epizootiology of *Pandora (= Erynia) neoaphidis* which would facilitate its practical exploitation for aphid control in field crops.

This was a Sustainable Arable Link project in collaboration with the University of Newcastle. Funding and in-kind support was provided by BBSRC, HGCA, HDC, PGRO, BPC, J. Sainsbury Plc, the Allium and Brassica Centre and NIAB. The monitor for the project was Richard Knight (FWAG).

The sections comprising this report are based on the three specific research areas which were;

1. Quantify the spatial and temporal spread of *P. neoaphidis* in field margins and adjacent crops and interpret this in relation to the epizootiology of the fungus and the opportunities for increasing fungus populations for aphid control.

1.1 Farm surveys and field margin sampling for entomopathogens

1.2 Modelling of infection transmission between *P. neoaphidis* and the pea aphid

1.3 Aerial dispersal and infection by *P. neoaphidis* conidia in agroecosystems

1.4 Studies on the role of stinging nettles as reservoirs of *P. neoaphidis*.

2. Quantify the impact of aphid physiology and behaviour on susceptibility to *P. neoaphidis* as influenced by host plant and insecticide resistance and interpret this in relation to fungus spread in populations.

2.1 Aphid susceptibility to *Pandora neoaphidis*

2.1.1 Pest aphid species

2.1.2 Non-pest aphid species

2.2 Plant and cultivar influence

2.2.1 Transgenic cultivar influence (Univ. of Newcastle collaboration)

2.3 Insecticide resistance

3. Develop discriminating "fingerprints" for field derived *P. neoaphidis* isolates with respect to their molecular and biological traits and use these to map fungus movement in the field.

3.1 *P. neoaphidis* species-specific primers

3.2 Molecular techniques to develop isolate-specific primers

3.3 Use of molecular fingerprint

3.4 Effects of fungicides on *P. neoaphidis*

3.5 Growth and enzyme characteristics of *P. neoaphidis*

Abstract

A four year project was undertaken to provide information on the epidemiology of the aphid pathogenic fungus *Pandora* (= *Erynia*) *neoaphidis* which would facilitate its practical exploitation for microbial control of aphids in field crops. In particular we focused on the potential for managed field margins as reservoirs of *P. neoaphidis* in agroecosystems.

During field studies conducted between 2000 and 2003, six new *P. neoaphidis* isolates were collected from primrose, pea and nettle aphids and included for evaluation in laboratory assays. Aphids on grasses such as Yorkshire fog, legumes (e.g. bird's-foot trefoil, clover) and stinging nettle, amongst others, were identified as beneficial sources of *P. neoaphidis* within agroecosystems. At an experimental field margin on Rothamsted Farm, the largest aphid and fungus densities were recorded in 2001, but numbers were generally small in all other years. In bean and wheat crops, conidia of *P. neoaphidis* were mostly found within 2 m of artifical release points but could be detected 12 m away. Pest aphids placed within 2 m of fungal sources became infected. Trials in polytunnels demonstrated that pest aphids on crop plants could be infected by spores carried downwind from infected aphids on non-crop plants such as nettles.

The overall ranking for laboratory susceptibility of pest and non-pest aphids to *P. neoaphidis* was: *Acyrthosiphon pisum* > *Aphis fabae, Microlophium carnosum, Metopolophium dirhodum, Myus persicae*, *Uroleucon jaceae* > *Sitobion avenae* > *Brevicoryne brassicae, Rhopalosiphum padi*. Isolates originally collected from non-pest aphids were able to infect pest aphids. There were no significant effects of host plant or cultivar (including a transgenic wheat cultivar expressing snowdrop lectin) on susceptibility of *A. pisum*, *M. dirhodum* or *M. persicae* to *P. neoaphidis*. Therefore, the host plant on which an aphid is feeding is unlikely to affect the performance of *P. neoaphidis*. Genotypes of *M. persicae* with high levels of esterase-based insecticide resistance were more resistant to fungal infection than insecticide-susceptible genotypes.

Species-specific molecular primers were developed for *P. neoaphidis* and the morphologically similar species *P. kondoiensis*. Intra-specific variation among *P. neoaphidis* isolates was quantified using ERIC, ISSR and RAPD PCR-based DNA fingerprint analyses. Three groups, or clusters, of *P. neoaphidis* isolates were established. Potential cluster- and isolate-specific diagnostic markers were identified and evaluated but require further development. Molecular studies supported laboratory bioassays which indicated free movement of *P. neoaphidis* isolates between different aphid species was possible. The optimal temperatures for growth of most *P. neoaphidis* isolates was between 18 and 22°C. Some isolates also grew reasonably well at 10-15°C. At recommended rates, chlorothalonil, dichlofluanid, fenhexamid, prochloraz and carbendazim completely inhibited conidia germination of *P. neoaphidis*. Mycelial growth of one isolate, originally from nettle aphid, was less affected by the fungicide azoxystrobin compared with three isolates from pest aphids.

In summary, the project has provided substantial field and laboratory data on the factors influencing the epidemiology of *P. neoaphidis* in patchy aphid populations in fragmented agricultural landscapes. Both molecular and biological attributes of fungal isolates suggest free movement of isolates between different aphid hosts in the field. Field margin plants with potential as reservoirs have been identified and cross transmission and dispersal of the fungus between aphid species occurring in margins and crops was demonstrated. This underpins the exploitation of field margins as reservoirs of *P. neoaphidis* for pest aphid control.

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Summary

The goal of this project was to provide information on the epidemiology of the aphid pathogenic fungus *Pandora* (= *Erynia*) *neoaphidis* which would facilitate its practical exploitation for microbial control of aphids in field crops. In particular we focused on the potential for managed field margins as reservoirs of *P. neoaphidis* in agroecosystems. The three main research aims were to (1) quantify spatial and temporal occurrence and spread of *P. neoaphidis* within and between field margins and associated crops, (2) assess factors affecting *P. neoaphidis* infection such as aphid species, host plant and insecticide resistance, and (3) determine molecular and biological traits to characterise different isolates, or strains, of *P. neoaphidis*.

Occurrence of *Pandora neoaphidis* in agroecosystems

Field surveys were conducted in crops, margins and hedgerows between 2000 and 2003 at various locations including a wildflower seed farm, pea and brassica fields, established margins at commerical farms (Colworth House, Beds. and Radcot Bridge Farm, Oxon.), and at an experimental field margin at Rothamsted Farm (Harpenden, Herts.). During field studies, six new *P. neoaphidis* isolates were collected from primose, pea and large nettle aphids for evaluation in laboratory assays. Several plant – aphid combinations were identified as useful sources of *P. neoaphidis* in agroecosystems. The dominant sources were *Sitobion* spp. on Yorkshire fog and *Microlophium carnosum* on stinging nettles. Other potentially beneficial plants with aphids and *P. neoaphidis* included crested dogstail, cocksfoot, bird's-foot trefoil, clover, teasel, bramble, and hogweed. However, low numbers of aphids were recorded during field studies and many more combinations may have been identified in years with larger aphid densities.

At the Rothamsted experimental field margin largest aphid densities in the margin (17 aphids per $0.25m^2$) were found during quadrat counts conducted in 2001. Average proportions of aphids infected or parasitised were less than 5% and 15%, respectively, in both the margin and adjacent wheat crop, except in 2003 when no infection or parasitism was recorded. (Figure 1).

There was some indication in the field margin that parasitism of aphids may also enhance fungal infection. However, conclusions on the seasonal and spatio-temporal interactions between aphids and *P. neoaphidis* were limited due to the small numbers of aphids found during the four years of field studies. Changes in the botanical composition of the experimental field margin were also assessed. From the original seed mix containing 33 plant species, a total of 14 species failed to establish. The optimal appearance of the margin was in 2001, when Yorkshire fog and crested dogstail were dominant grasses and this coincided with the largest aphid densities recorded during the four years of sampling. From 2002 onwards there was a deterioration in the quality of field margin plants which was illustrated by the presence of stinging nettle, thistles and sorrel which gave the margin an atypical appearance.

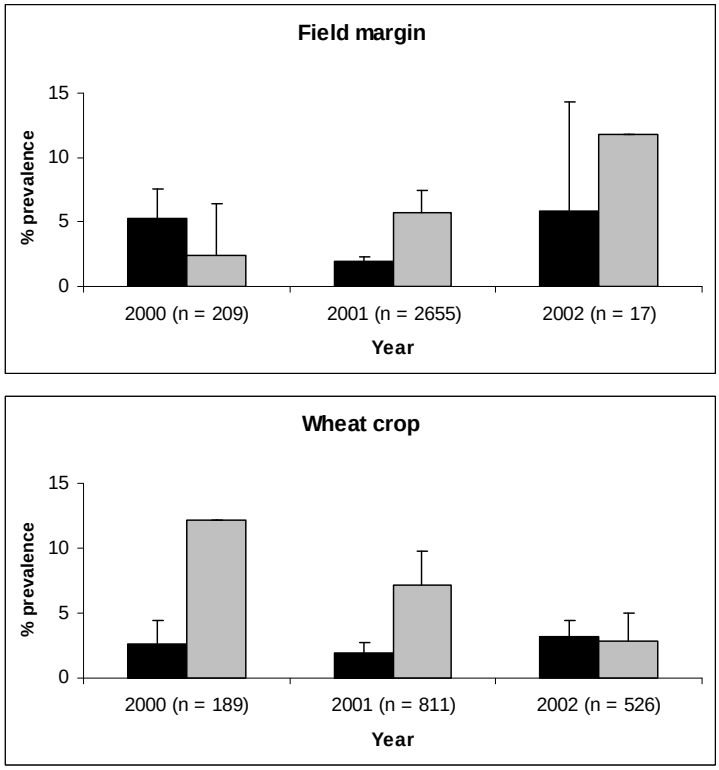

Figure 1: Mean prevalences of fungal infection (black bars) and parasitism (grey bars) in aphids from Stubbings field margin and wheat crop. Numbers of aphids in parentheses. No infection or parasitism was recorded in 2003. Bars represent SE, omitted for parasitism in margin in 2002 (SE = 30.06) and in crop in 2000 (SE = 6.94).

Supplementary sampling studies were also made at two sites of stinging nettles. Proportion of *M. carnosum* infected in populations under an oak tree and in the perimeter of a cereal field at Rothamsted varied between 20-30% in 2000 and 2002, but <10% infection was recorded in 2001 and 2003 (Figure 2). Parasitism was <5% at both sites. The dominant fungus was *P. neoaphidis* but other species, such as *Entomophthora planconiana* and *Neozygites microlophii*, were also recorded suggesting nettle aphid may be an important host for entomophthoralean fungal communities in agroecosystems. Peak infection of *M. carnosum* occurred from mid-June at both sites, representing a potentially important early-season source of fungal infection for biological control of pest aphids establishing in adjacent crops at this time.

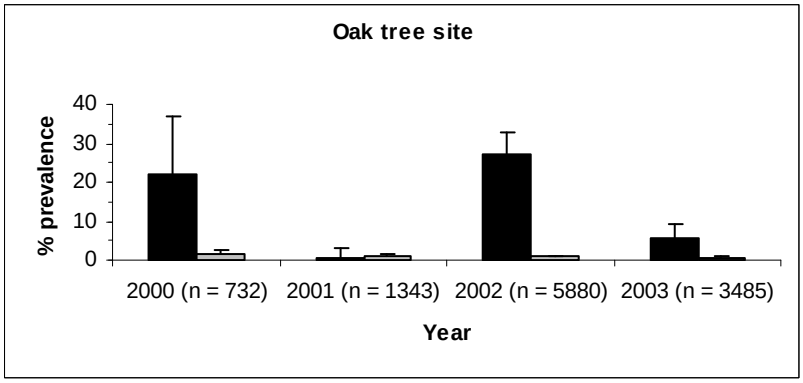

Figure 2: Mean prevalences for fungal infection (black bars) and parasitism (grey bars) of nettle aphids from Stubbings oak tree nettle site. Numbers of aphids in parentheses.

Spread of *Pandora neoaphidis* within and between aphid populations in agroecosystems

Four sets of experiments were done to evaluate spread at different spatial scales; (i) modelling of transmission and infection, (ii) aerial dispersal of spores from nettle aphids to adjacent pest aphids and initiation of infection, (iii) aerial dispersal of spores and initiation of infection withinin field crops, and (iv) infection in field cages.

(i) A series of pot plant and field experiments were done to estimate transmission of *P. neoaphidis* in pea aphid populations of different densities. These data will be used to parameterise an arthropod - microorganism mathematical model which could ultimately be used to help us predict the host and fungus densities necessary to initiate epizootics in field crops. Preliminary results indicate that rate of transmission is not greatly influenced by inoculum density.

(ii) In a polytunnel experiment, conidia of *P. neoaphidis* from sporulating aphid cadavers on nettle plants were able to initiate infections in aphid colonies of *Acyrthosiphon pisum* or *Metopolophium dirhodum* on potted plants of bean or barley, respectively, located downwind from the nettle source (1m). This indicated the potential for cross-infection from non-pest to pest aphids on a small-scale.

(iii) and (iv) In a field experiment, conidia dispersal from laboratory produced fungal material placed in bean and wheat crops was assessed using rotary samplers ("rotorods") powered by electric motors. Conidia were mostly caught within 2 m of inoculum sources but could be detected 12 m away from sources within both crops and were likely to have travelled further than this. Dispersal of conidia did not follow the general wind direction, indicating the influence of gusts and eddies on transport and distribution of fungal conidia. Between 5 and 15% of sentinel *A. pisum* and *M. dirhodum* aphids placed within 2 m of inoculum sources became infected in both crops (Figure 3).

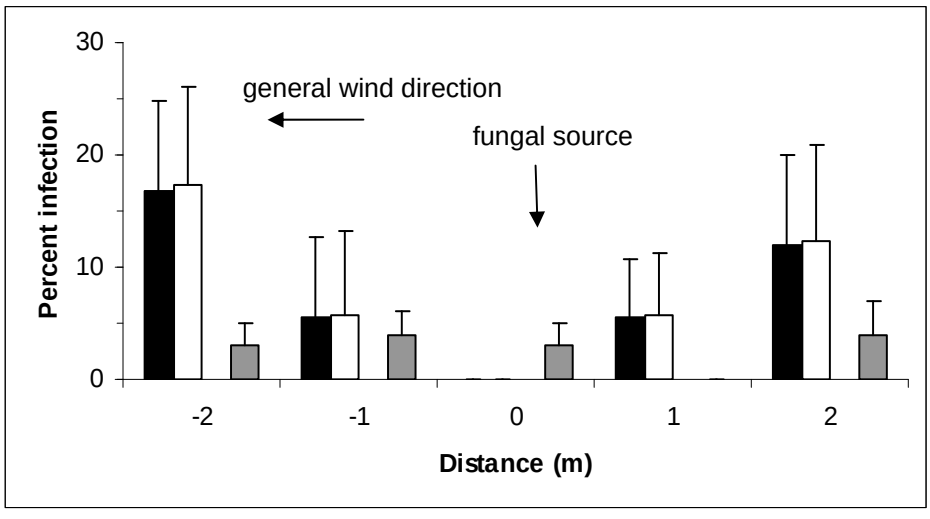

Figure 3: Mean infection (+ SE) by *P. neoaphidis* in sentinel aphids placed at different distances from mycelial mats. *Acyrthosiphon pisum* was exposed overnight in a bean crop on 2-3 August (■) and 3-4 August (□), while *Metopolophium dirhodum* was exposed in a wheat crop on 2-3 August (no infection) and 3-4 August (▒).

For both the polytunnel and field experiment, plant architecture may have played a significant role in infection as the broader leaves of bean plants may have captured more conidia than the leaf blades of barley plants. Also, for both experiments, aphid colonies were only subjected to a single night of exposure to inoculum. Infection would be much greater with longer exposure periods resulting in several cycles of infection. However, dispersal and the ability to cause infection was demonstrated. In a separate study, attempts to initiate and monitor epizootics in caged bean and wheat crops were unsuccessful because of the poor quality of laboratory produced fungus. The lack of optimal methods for mass producing highly infective fungal inoculum is a key constraint in the use of *P. neoaphidis* as a bioinsecticide.

Factors affecting aphid susceptibility to *P. neoaphidis*

Laboratory bioassays were used to determine the (i) variation in susceptibility of seven pest aphid species to different isolates, (ii) differences in susceptibility between pest and non-pest aphids, (iii) effects of host plant and cultivar (including a transgenic variety), and (iv) influence of insecticide-resistance in *M. persicae* clones on susceptibility. In all bioassays sporulating fungal mycelium or infected aphid cadavers were placed above batches of apterous adults to inoculate them. Aphids were exposed to conidia "showers" for different time periods to achieve different doses, incubated on clean plants and mortality assessed for up to seven days. In all experiments, bioassays were repeated on three separate occasions.

(i) and (ii) A novel system was devised for comparing different isolates of *P. neoaphidis* against *A. pisum, Aphis fabae, Brevicoryne brassicae, Myzus persicae, M. dirhodum, Sitobion avenae* and *Rhopalosiphum padi*. From screening assays, 16 of 20 *P. neoaphidis* isolates, including those collected from non-pest aphids such as nettle aphids, were able to initiate moderate to high levels of infections (40-100%) in all seven pest species. Dose-response assays with three isolates (NW 314, NW 316 and NW 327) gave median lethal doses (LD_{50}) of 8 - 105 conidia mm $^{-2}$ against *A. pisum*, 38 - 92 conidia mm $^{-2}$ against *M. dirhodum* and 105 - 626 mm $^{-2}$ against *M. persicae*. The LD_{50} values were then tested as discrimatory doses against the remaining aphid species with *M. persicae* as a reference species, from which *B. brassicae* and *R. padi* were the least susceptible to infection. The overall ranking of aphids to infection by *P. neoaphidis* was: *A. pisum* > *A. fabae, M. dirhodum, M. persicae, S. avenae* > *B. brassicae, R. padi*. Also, an isolate originally from *A. pisum* was still able to cause infection in this species and *M. dirhodum* after passage through the nettle aphid. In separate experiments involving two non-pest aphids, susceptibility of the nettle aphid, *M. carnosum*, was similar to *M. dirhodum* while susceptibility of *Uroleucon jaceae* on knapweed was similar to *M. persicae*. The results show that *P. neoaphidis* can cross-infect aphid species which occur both in field crops and non-cropped areas, such as field margins. This is an important feature for the development of conservation biological control strategies where it is essential that inoculum produced in margin aphids is infective to pest aphids in the adjacent crop. The work also indicated the relative importance of different aphid species as sources of P. neoaphidis, e.g. *A. pisum* and *M. carnosum* are likely to be more important than *U. jaceae*.

(iii) There was a non-significant trend for the percentage of aphids infected to be greater on original than alternate host plant species for *A. pisum* (bean compared to pea, respectively), *M. dirhodum* (barley compared to wheat) and *M. persicae* (Chinese cabbage compared to potato). Infection levels also varied among aphid species, from *ca*. 20% for *A. pisum*, 12% for *M. dirhodum*, and 5% for *M. persicae*, supporting the earlier susceptibility assays. There were also no statistical differences between percentage infection for aphids feeding on original or alternate plant varieties for *A. pisum* (beans; "The Sutton" compared to "Victor"), *M. dirhodum* (barley; "Gleam" compared to "Regina") or *M. persicae* (Chinese cabbage; "Wongbok" compared to "Kasumi"). The mean proportion becoming infected was *ca*. 90% for *A. pisum*, 14% for *M. dirhodum* (lower than expected) and 50% for *M. persicae* (higher than expected) (Figure 4).

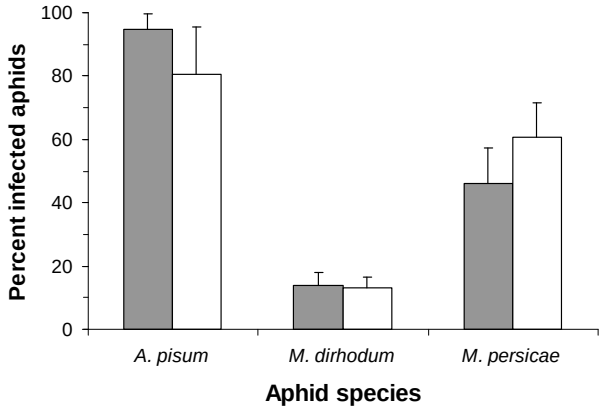

Figure 4: Mean percent infection (+SE) by *P. neoaphidis* averaged over three bioassay runs for each of three aphid species on original (grey bars) or alternate (white bars) plant varieties.

In collaboration with Dr AMR Gatehouse, University of Newcastle, the susceptibility of *M. dirhodum* on a transgenic wheat cultivar expressing snowdrop lectin (GNA) was evaluated. This lectin can confer partial plant resistance against sucking pests such as aphids. Expression levels in the transgenic line were approximately 0.1% total soluble protein, with expression being greater in younger tissues. The percentage of aphids infected by *P. neoaphidis* did not differ significantly on transgenic GNA and non-transformed lines (91% and 82%, respectively; Figure 5). Also, fecundity did not differ between control and infected aphids on either line and was *ca*. 18 nymphs aptera^{-1} over the seven day monitoring period. Time to kill was *ca*. five days for *M. dirhodum* on both varieties in two of the three assays. The results suggest that transgenic wheat expressing GNA would not affect the performance of *P. neoaphidis*. It also appeared that *M. dirhodum* was unaffected by GNA, since there were no differences in either aphid mortality or fecundity between transgenic and untransformed wheat lines. Further studies are needed to determine GNA levels actually present in the phloem of crop plants, as well as the potential susceptibility of a much wider range of aphid species to GNA than previously considered.

All of these bioassays indicated that there were no significant effects of host plant species or variety on *P. neoaphidis* performance. Hence, transmission of *P. neoaphidis* between aphids in crop and non-crop areas is unlikely to be affected by aphid food plant. As no differences in susceptibility related to host plant were found, these experiments were not followed up with behavioural studies on conidia acquisition.

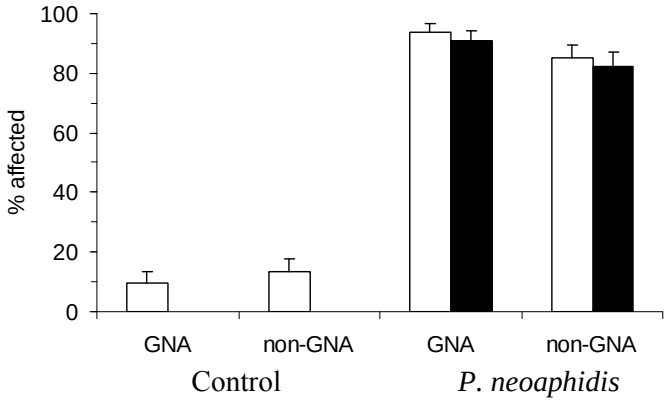

Figure 5: Mortality (□) and infection (■) for *Metopolophium dirhodum* on trangenic GNA or non-transformed wheat lines, either with or without inoculation by *Pandora neoaphidis* conidia. There was no infection in uninoculated groups of aphids. Means and SE pooled from three bioassays.

The susceptibility of ten different genotypes of *M. persicae* to *P. neoaphidis* were also assesed. Aphid genotypes were chosen according to insecticide resistance mechanisms - either esterase-based or knock-down (kdr) resistance. From dose response assays there were no significant differences in the virulence of two isolates to four insecticide-susceptible genotypes. Further experiments comparing eight genotypes with one isolate showed that virulence was greatest against the insecticide-susceptible genotypes and least against *M. persicae* genotypes with high levels of esterase - based resistance. The results suggest there is variation in responses of insecticide-susceptible genotypes to infection by *P. neoaphidis*, but also that aphids with a high level of esterase resistance are also more tolerant to *P. neoaphidis* infection. Further studies are needed to determine if the outcome from this work, using the highest level of resistance, are also consistent with different resistance levels and genotypes of *M. persicae*. Attempts to develop protocols for evaluating the role of behaviour in susceptibility of different genotypes and transmission in mixed populations were not successful due to the overall moderate - low susceptibility of *M. persicae*, with LD_{50} values of 200-1000 conidia mm^{-2} for all genotypes.

Molecular characterisation of *P. neoaphidis* isolates

Genetic variation was assessed amongst 37 isolates of *P. neoaphidis* from a range of pest and non-pest aphid species, as well as 21 isolates from eight other entomophthoralean species. Universal primers were used to amplify the internal transcribed spacer (ITS) ribosomal DNA (rDNA) regions and all of the species tested produced discrete ITS groups, with the exception of *Conidiobolus* spp. Neighbour-joining analysis of the ITS2 regions from *P. neoaphidis*, *P. kondoiensis* and *Zoophthora radicans* demonstrated that these three species formed distinct groups with sequence identities of 58 - 82% amongst the groups. An ITS size of 1100 bp was diagnostic for *P. neoaphidis*, while 1450 bp was characteristic of the closely related aphid pathogen *P. kondoiensis*. ITS-RFLP analysis was useful in distinguishing between different entomophthoralean species but was not sufficiently discrimitory for producing *P. neoaphidis* isolate-specific molecular markers. Our studies also indicated that *P. kondoiensis* may perhaps be associated with cereal aphids in Europe

although no definite recordings of the species have been made in the UK. Sequencing analysis of the complete ITS region from *P. neoaphidis* and *P. kondoiensis* allowed species-specific primers to be developed for these two species, which were successfully used to screen aphids infected in laboratory bioassays and from field collected samples, without prior isolation of the fungus. Discrimination between the two species is important as *P. kondoiensis* may be an important early season pathogen of cereal aphids in France, and hence potentially also in the UK.

Within-species genetic diversity was investigated using ISSR (Inter Simple Sequence Repeat), ERIC (Enterobacterial Repetitive Intergenic Consensus), RAPD (Random Amplification of Polymorphic DNA) primers and a random primer based on the universal M13 core sequence. Thirty isolates of *P. neoaphidis* and six isolates from other entomophthoralean species were evaluated and results indicated that *P. neoaphidis* isolates were variable but could be separated into a single monophyletic group with three sub-groups. UK isolates occupied two of these three sub-groups or clusters along with other isolates with a worldwide distribution. One of these consisted of four out of five isolates obtained from the large nettle aphid, *M. carnosum* (Figure 6). No other relationships with aphid species or geographic origin were identified supporting laboratory bioassays which indicated that *P. neoaphidis* should be ecologically mobile and able to cross-infect several aphid host species.

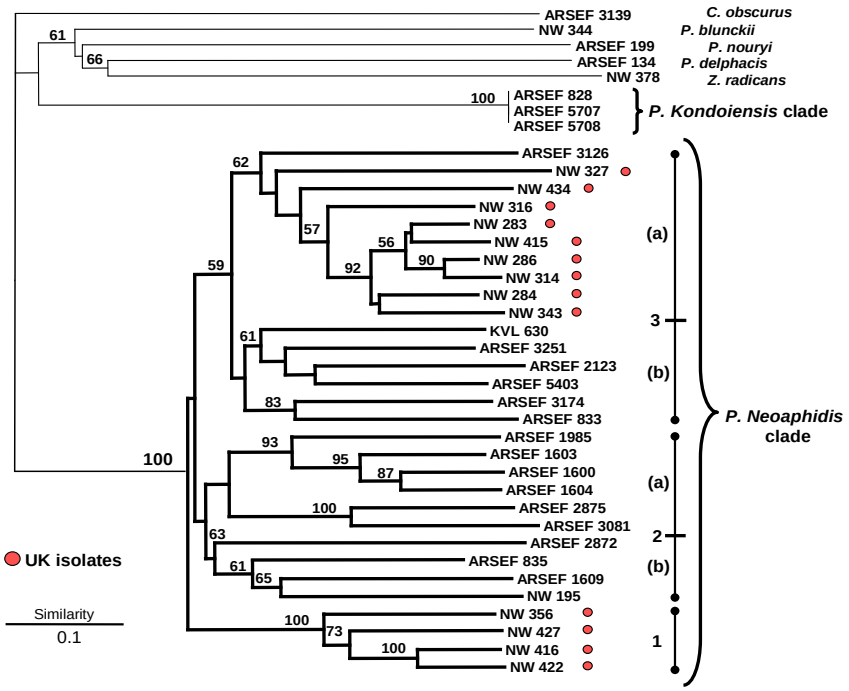

Figure 6: Phylogenetic relationships inferred from Neighbour-Joining analysis from a similarity matrix created using Jaccard's co-efficient, of the presence or absence of PCR products. The branches connecting the *P. neoaphidis* isolates are shown with bolder lines. Sub-clades are indicated by numbers.

Polymorphic bands that were unique to a particular *P. neoaphidis* isolate or cluster (Figure 7), were excised from gels, reamplified, cloned and sequenced to identify potential isolate or cluster specific primers, which would be useful for isolate identification within field collected aphids. Fourteen primers were

produced and tested individually or in pairs against representative isolates from each cluster. However, only one pair of primers showed potential to discriminate between isolates. These primers (and additional primers) require further developmental work and optimisation before they can be used to identify particular isolates in infected aphids. Infected aphids collected from the field in 2002 were preserved for testing with appropriate primers should they have become available. Virtually no infection by *P. neoaphidis* was found in aphids during 2003 (the year when it was planned to use the isolate specific primers) because of the exceptionally hot conditions during the spring and summer months.

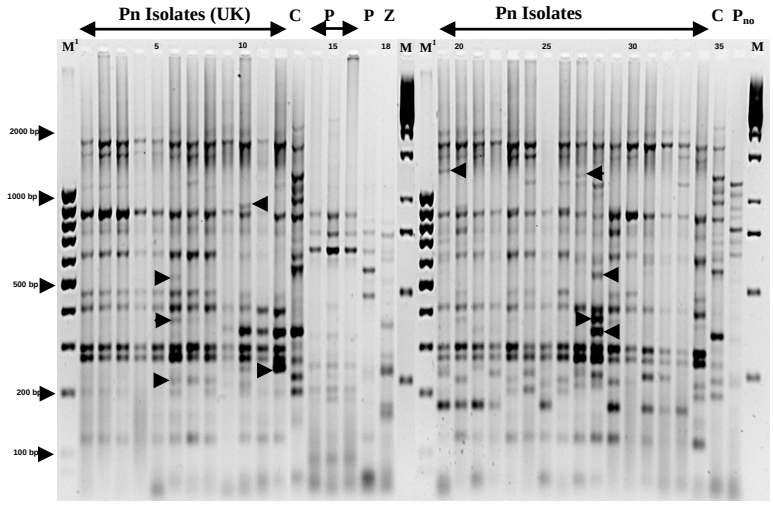

Figure 7: ERIC-PCR fingerprints of *P. neoaphidis* isolates and other related entomopathogenic fungi. Arrows within gel lanes indicate a range of polymorphic bands between *P. neoaphidis* isolates.

Other biological traits of *P. neoaphidis* isolates

The effects of fungicides, temperature and nutrient media on different isolates of *P. neoaphidis* were evaluated. In Petri dish assays, azoxystrobin, carbendazin and prochloraz completely inhibited mycelial growth at 100 ppm, while there was 20-40% inhibition with a further seven fungicides tested (epoxiconazole, chlorothalonil, dichfluanid, maneb, fenhexamid, fenpropimorph and metalaxyl). Stimulatory effects on mycelial growth were found at 1 ppm with dichlofluanid, maneb and metalaxyl. The fungicides chlorothalonil, dichlofluanid, fenhexamid, prochloraz and carbendazim caused complete inhibition of conidia germination at recommended rates. There was evidence for a dose-response effect in mycelial inhibition of different isolates to azoxystrobin. Of the four isolates tested (NW 286, NW 305, NW 314 and NW 356), NW 356 was least affected at 0.1 and 1 ppm azoxystrobin and exhibited some growth at 10 ppm. Overall, isolates varied in their sensitivity and possible adverse effects depended on the chemical and on the life stage of *P. neoaphidis*.

Seventeen isolates of *P. neoaphidis* and one isolate of *P. kondoiensis* were grown on standard agar medium (Sabouraud dextrose agar supplemented with milk and egg yolk, SEMA) at five temperatures from 4 - 30°C. Colony diameters were measured at regular intervals and data summed to produce accumulated growth over time for each isolate at each temperature. All isolates were unable to grow at 30°C and there was limited growth at 4°C. The optimal temperatures for growth of the for the majority of isolates was between 18 and 22°C. At the optimum temperatures of 18 – 22 °C, isolates separated into those which grew

very fast, those which were much slower and those which had intermediate growth rates. The majority of isolates fell into the intermediate group, with only a few at the extremes. Some isolates also grew reasonably well at 10-15°C. This may be an indication that season long activity of *P. neoaphidis* may occur due to a succession of different isolates with differing temperature optima and host virulence. *Pandora kondoiensis* was unable to grow at 25°C in contrast to several of the *P. neoaphidis* isolates. Growth on a low nutrient semi-defined agar medium (SDEM) at 20°C was greatest for *P. kondoiensis*. Although only one *P. kondoiensis* isolate was used, it had better growth at low temperatures and on comparitively nutrient poor media than the *P. neoaphidis* isolates. This may indicate enhanced activity early in the season and a different host range.

Semi-quantitative analysis of 19 enzymes using the proprietary Api-Zym kit (BioMérieux, France) revealed some differences between intra- and extra-cellular enzymes produced by seven isolates of *P. neoaphidis* and one isolate of *P. kondoiensis* (Figure 8). Isolates were grown in liquid culture, mycelium removed and the culture filtrate evaluated for extra-cellular enzymes. Intracellular enzymes were released by disrupting hyphae and resuspending the contents prior to evaluation. Colour reactions were scored against a standard colour scale which ranged from 0 to 5. The most important differences between isolates related to enzymes involved in carbohydrate and protein degradation. The production of chitinases, lipases and proteases, which help break down aphid cell walls may play a role in characterising the virulence of the different *P. neoaphidis* isolates. Further work is needed to understand changes in Api-Zym profiles pre- and post-infection with different isolates, which may provide a better prediction of field effectiveness.

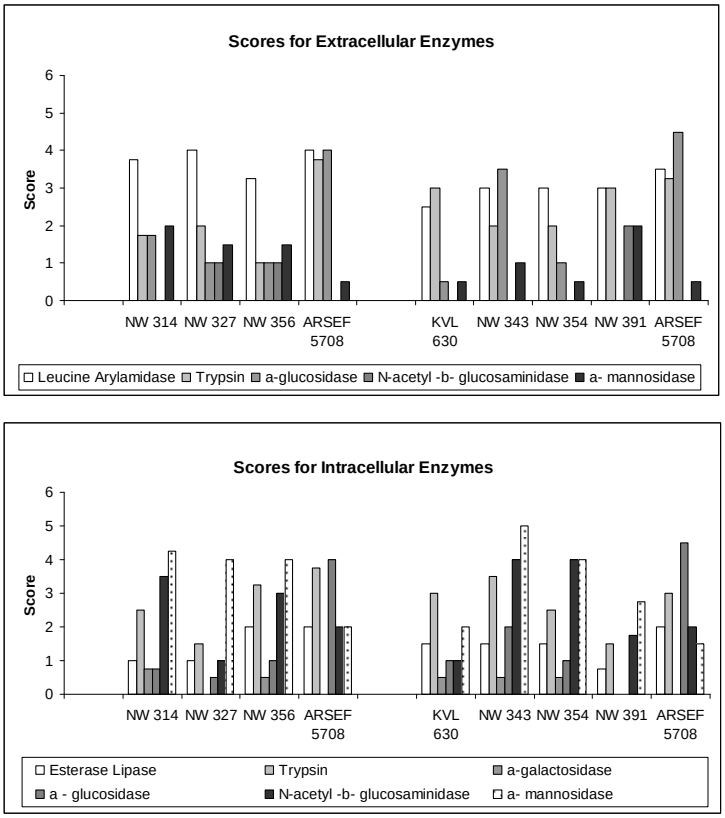

Figure 8: Selected differences in enzyme activities with different *Pandora* isolates using Api-Zym on two different occasions

Principal conclusions and opportunities

The project provided unique results on the relative susceptibility of nine aphid species to infection by different *P. neoaphidis* isolates, the influence of transgenic proteins on aphid susceptibility, interactions between insecticide resistance and fungal infection and species specific primers to distinguish between *P. neoaphidis* and *P. kondoiensis*. These areas were investigated for the first time and represent a significant contribution to international research on insect/ pathogen interactions.

Specifically, laboratory bioassays demonstrated inherent variation in susceptibility amongst different aphid species. Highly susceptible species are likely to be useful sources of infection when they are in margins (e.g. *A. pisum* and *M. carnosum*) and are also likely to be key species for the survival of *P. neoaphidis* in agroecosystems. In our assays aphid susceptibility was not affected by host plant species or variety, including a transgenic wheat line. This suggests that *P. neoaphidis* will be able to disperse between aphids of the same species feeding on non-crop and crop plants (e.g. *A. pisum* and *M. dirhodum*). Further work is required using manipulative replicated experiments with the plant mixtures identified in this project, in order to determine the full potential for multiplication and spread of *P. neoaphidis* from margins into adjacent crops. In addition research is required on appropriate margin management techniques to optimise conservation of *P. neoaphidis* within agroecosystems.

An important achievement in this project was the development of species specific primers to distinguish between *P. neoaphidis* and *P. kondoiensis* infections in aphids. The latter species has not been recorded in the UK but is morphologically very similar to *P. neoaphidis* and may have been misidentified. The molecular markers will not only ensure accurate identification but also permit fundamental studies on inter-specific competition for aphid hosts between the two species. Primers capable of identifying particular isolates/ genotypes within infected hosts were not developed in the duration of the project. Had they been available, it would have been possible to identify the spatial occurrence of isolates/ genotypes in aphids from field margins and adjacent crops. From this it may have been possible to infer dispersal between margin "sources" and crop "sinks" as well as the temporal succession of isolates in different habitats through the season. However, likely primers were identified and still have the potential for development as specific markers but require further work.

Analysis of data from molecular and biological (pathogenicity, temperature, enzyme, spore morphology) studies is in progress and will be used to determine 'ecological fingerprints' to characterise groupings of isolates or genotypes with similar attributes.

In summary, the project has provided substantial field and laboratory data on the factors influencing the epidemiology of P. neoaphidis in patchy aphid populations in fragmented agricultural landscapes. Both molecular and biological attributes of fungal isolates suggest free movement of isolates between different aphid hosts in the field. Field margin plants with potential as reservoirs have been identified and cross transmission and dispersal of the fungus between aphid species occurring in margins and crops was demonstrated. This underpins the exploitation of field margins as reservoirs of *P. neoaphidis* for pest aphid control.

Objective 1 Quantify the spatial and temporal spread of *Pandora neoaphidis*

1.1 Farm Surveys and Field Margin Sampling for Entomopathogens

Abstract

Studies were carried out between 2000 and 2003 on the occurrence of the aphid-specific fungal pathogen *Pandora neoaphidis* in field margins and other crop and non-crop areas. During surveys, six new isolates were collected for evaluation in laboratory assays. A preliminary appraisal was made on plants and aphid species which may be beneficial as sources of *P. neoaphidis* in agroecosystems. These include grasses such as Yorkshire fog, legumes (e.g. bird's-foot trefoil, clover) and stinging nettle. Detailed sampling studies were conducted at an experimental field margin at Rothamsted. Largest aphid densities in the margin (17 aphids $0.25m^{-2}$) were found during 2001 but peak numbers only occurred for two weeks. Conclusions on the seasonal and spatio-temporal interactions between aphids and *P. neoaphidis* were limited due to the low numbers of aphids found during the four years of field studies. Changes in the botanical composition of the experimental field margin were also assessed. From the original seed mix containing 33 plant species, a total of 14 species failed to establish, possibly due to soil, pest and site-specific factors. Some aspects for future research are given, especially concerning margin management techniques.

Introduction

Field margins may be potential sources of aphid-pathogenic fungi, especially *Pandora neoaphidis* (Zygomycetes: Entomophthorales). However, there appears to be no information on aphid-pathogenic fungi in arable margins and very little data on these natural enemies in non-cropped areas such as hedgerows. Field studies were performed in order to (i) obtain new isolates of *P. neoaphidis* for evaluation in concurrent laboratory studies on virulence and molecular biology, (ii) identify plant-aphid combinations which could be recommended for field margins and (iii) to obtain baseline data on the occurence of *P. neoaphidis* and similar fungal pathogens in field margins and on crop and non-crop plants.

Three sets of studies were done; First, one-off surveys were carried out at various sites; Second, new and established grass and wildflower field margins were surveyed at two farms in Bedfordshire and Oxfordshire; and finally, an experimental field margin was established and used at Rothamsted Farm for intensive sampling studies on aphid-entomopathogen interactions.

Methods and Materials

Single surveys in 2000

Three visits were made to different locations for the purpose of finding aphids infected by *P. neoaphidis* for isolation and use in laboratory studies. Surveys were done at Herbiseeds (Wokingham, Berks., grid ref. SU 829 722) on 8th June, a maturing organic pea crop near Kirton (Bush Lane Field no. 159, Algarkirk Grange, Kirton, Lincs., grid ref. TF 314 346) on 19th July, and brassica fields near the Allium and Brassica Centre Ltd. (Kirton, Boston, Lincs.) on 31st October. At Herbiseeds, visual inspections were made

of various blocks of flowers and grasses cultivated for seed. In addition, hedgerow plants were also investigated for aphids and fungal infection. The pea crop was visited specifically in order to obtain infected pea aphids for laboratory isolation, on the advice of the Processors and Growers Organisation (PGRO). Brassica crops around Kirton were examined for aphids and infection.

Surveys at Colworth House and Radcot Bridge Farm

Sampling and surveys were done at weekly or two weekly intervals at two farms between June and July in 2000 and 2001. At Colworth House (Sharnbrook, Beds., grid ref. SP 980 600) a newly prepared 6m flower margin, sown in March 2000, and two 20 m wide grass conservation headlands were sampled. At Radcot Bridge Farm (Radcot, Oxon., grid ref. SU 270 995) one 6 m wide flower margin and one 6 m wide grass margin were regularly studied during farm visits, along with small plots used for experiments on herb-rich mixtures (designated W11 and W13 on 1998/99 Agroecology trial plans).

Field margin sampling was carried out using 0.25 m^2 quadrat counts of live and dead aphids every 8-10 m in a 30 m transect within each field margin, and a total of 30-40 counts were made in each margin. Samples of live aphids, parasitoid mummies and fungus-infected cadavers were collected whenever they were encountered. Crop sampling was done by non-systematic searches in cereal and oilseed rape crops within 20-50 m of the field margins.

Stubbings field, Rothamsted Farm

In 2000, one edge of a field boundary was prepared for conversion into a field margin at Rothamsted Experimental Station (Ordnance Survey grid ref. TL 134 135). This experimental margin was 80 m in length, 5 m in width, with a 1 m perennial ryegrass strip between the margin and the adjacent woodland, as well as a 1 m sterile strip produced by soil cultivation between the margin and the wheat crop. The area had previously contained emerged winter wheat, which was removed mechanically and cultivated to form a fine seed bed in late-February 2000. The seed mix had been ordered in consulation with the suppliers (Herbiseeds, Wokingham, Berks.), and was based on a conservation headland mix with some additions to account for the degree of shadiness caused by overhanging tree branches in the margin site, and also with certain plant species from the literature considered to be beneficial for aphids and therefore potentially for *P. neoaphidis*. The seed mix was hand-sown in March 2000, and an electric fence was positioned around the margin to prevent feeding by rabbits. There was a delayed emergence because of dry spring conditions but establishment of plants was satisfactory by June 2000.

Plastic flexicanes were used to mark fixed sampling positions within the margin and the wheat crop (Figure 1.1). Quadrat counts (0.25 m^2) were performed in the margin in two lines of 30 counts, except in 2000 when only 30 counts were taken from the central area of the margin. During counts, aphid numbers and stages (apterous adults, alate adults, nymphs) feeding on different plants were noted, as well as the numbers of infected cadavers or parasitised mummies. Samples of living alate aphids, cadavers and mummies were taken for identification. Plant assessments were made in mid- or late-July each year, by noting the presence or absence of plant species in all of the quadrats.

Crop sampling was perfomed in seven lines marked at right angles to the margin. Lines were 10 m apart and each line had nine sample points (Figure 1.1). At each point, five wheat tillers were selected and counts of aphids, cadavers and mummies were made by examining the stems, leaves and ears of each of the tillers.

Data on numbers of live, infected and parasitised aphids were summed for all sample dates for each year before logistic regression analyses were conducted using the Generalized Linear Models procedure in GenStat (GenStat Committee, 2002). The aim was to determine if their were differences between years for prevalence of infection or parasitism in either the field margin or the wheat crop. Prevalence is defined as the numbers of new and old cases of a disease (or other natural enemy activity) recorded on successive sampling dates (Fuxa and Tanada, 1987). Analyses involving repeated measures (sample date, sample position) were not performed because of a large number of zero values. Data for 2001 were also analysed to determine spatio-temporal associations between aphids, fungal infections and parasitism using the SADIE software developed at Rothamsted (Spatial Analyses by Distance IndicEs; Winder *et al.*., 2001).

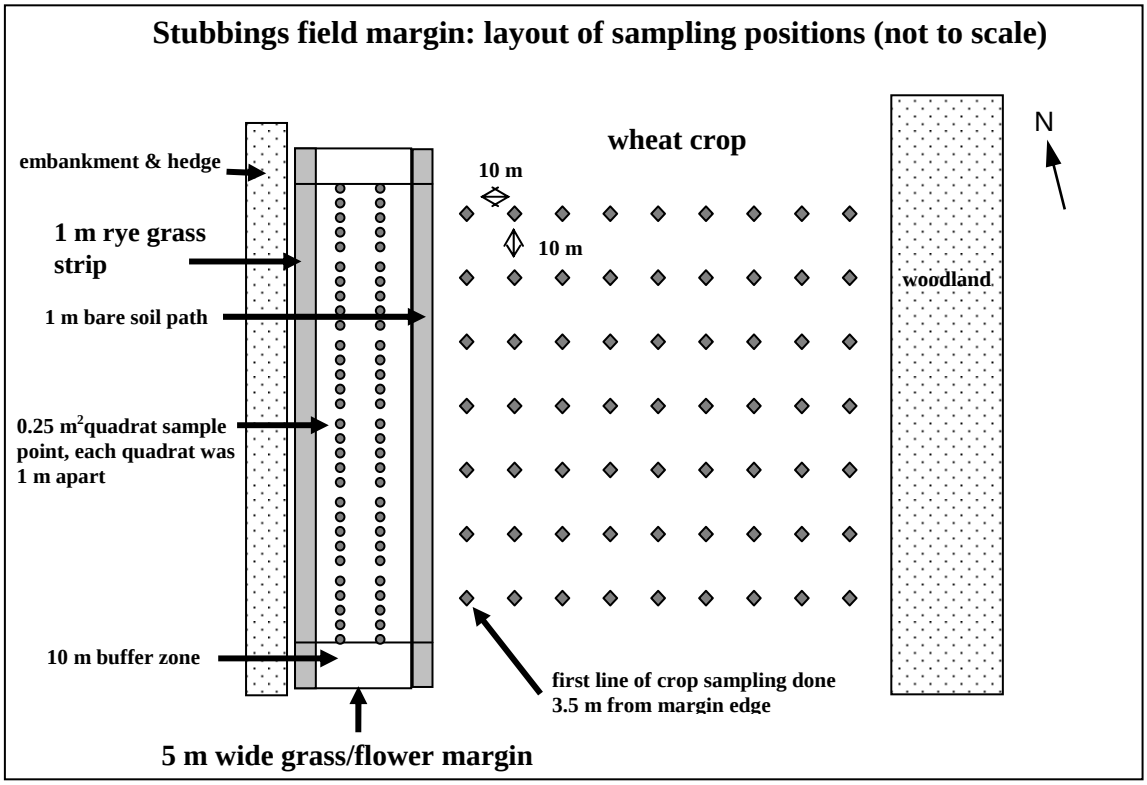

Figure 1.1

Schematic arrangement of sampling positions at Stubbings field margin (total length 80 m, width 6 m). Two lines of quadrats, 2.0 m apart, were marked out in the margin. Each line was 1.5 m from the margin edge. In 2000, only one line of 30 quadrats located centrally within the margin was used for sampling. Crop sampling was carried out by examination of five wheat tillers (heads, leaves, stems) at each intersection of a 7 X 9 grid.

Results

Collect isolates

A total of six successful isolations of *P. neoaphidis* were made from aphids collected during field surveys and studies (Table 1.1). The isolates were evaluated in virulence and molecular studies against a range of other isolates from Rothamsted and other culture collections (see Objectives 2.1 and 3.1).

Table 1.1: Isolates of *Pandora neoaphidis* collected during field studies, 2000-2003

Code no.	Aphid host	Common name	Location	Year
NW 415	*Microlophium primulae*	Primrose aphid	St. Albans, Herts.	2000
NW 416	*Microlophium carnosum*	Large nettle aphid	Rothamsted Farm, Herts.	2000
NW 422	*Microlophium carnosum*		Rothamsted Farm, Herts.	2000
NW 427	*Microlophium carnosum*		Colworth House, Beds.	2000
NW 434	*Acyrthosiphon pisum*	Pea aphid	Rothamsted Farm, Herts.	2001
NW 438	*Acyrthosiphon pisum*		Rothamsted Farm, Herts.	2001

All isolates are preserved in the fungal entomopathogen culture collection at Rothamsted Research.

Identify plants

During the course of the work, certain aphid-plant combinations were considered to have potential for enhancement of *P. neoaphidis* in agroecosystems (Table 1.2). The most common combinations for *P. neoaphidis* were *Sitobion* spp. on Yorkshire fog and *Microlophium carnosum* on stinging nettles. However, there were low numbers of aphids during field studies conducted between May and October during each of the four years of the project. In years with larger aphid densities, many more combinations may have been found. Nettle aphid, *M. carnosum*, was also a good host for other entomophthoralean fungi (Table 1.3), and may be important in generally maintaining biodiversity of insect-pathogenic fungi in agroecosystems.

Table 1.2: Assessment of plant-aphid combinations for enhancement of *P. neoaphidis* in agroecosystems

Plant	Category	Aphid species
Grasses		
Yorkshire fog	Very good	*Sitobion* spp.
Crested dogstail	Good	*Sitobion* spp.
Cocksfoot	Good	*Sitobion* spp.
Wildflowers		
Bird's-foot trefoil	Good	*Acyrthosiphon pisum*
Clovers	Good	*Acyrthosiphon pisum*
Knapweed	Poor	*Uroleucon* spp.
Teasel	Good	*Macrosiphum rosae*
Hedgerow plants		
Bramble	Good	*Amphorophora rubi*
Hogweed	Good	*Cavariellia* spp.
Nettle	Very good	*Microlophium carnosum*
Farm "weeds"		
Cleavers	Poor	*Aphis fabae* group
Creeping thistle	Poor	*Aphis fabae* group, *Uroleucon* spp.
Sowthistles	Good	*Hyperomyzus lactucae*

Monitor margins

Single surveys in 2000

Infected aphids were found on wildflowers and from a mature pea crop (Table 1.3), but no infections were detected from aphids feeding on brassica crops. During surveys and sampling studies, infected aphids were usually found as single individuals, not in colonies with large numbers of diseased aphids, and infections were two to four days old, which limited attempts for succesful laboratory isolations.

Table 1.3: Records of aphid species and fungal infections obtained from various sites during field studies.

Site	Plant	Aphid host	Fungal pathogen
Herbiseeds, Berks.	Common centaury ?	*Macrosiphum rosae?*	*Pandora neoaphidis*
	hogweed	*Cavariella pastinaceae* *Cavariella theobaldi*	*Pandora neoaphidis* *Entomophthora planchoniana*
	giant hogweed	*Cavariella theobaldi*	*Pandora neoaphidis*
Algarkirk, Lincs.	pea crop	*Acyrthosiphon pisum*	*Pandora neoaphidis*
Radcot Bridge Farm, Oxon.	nettle	*Microlophium carnosum*	*Pandora neoaphidis*
	oilseed rape	*Brevicoryne brassicae*	*Pandora neoaphidis*
	teasel	*Macrosiphum rosae*	*Pandora neoaphidis*
	nettle	*Microlophium carnosum*	*Pandora neoaphidis*
	unknown tree	*Phorodon humuli*	*Pandora neoaphidis*
	cleavers	*Aphis fabae* group	*Pandora neoaphidis*
	knapweed	*Uroleucon* sp. ?	*Entomophthora planchoniana*
	grass plots	*Macrosiphoniella* sp. ?	*Entomophthora planchoniana*
Colworth House. Beds.	nettle	*Microlophium carnosum*	*Pandora neoaphidis*
	prickly sowthistle	*Hyperomyzus lactucae* *Uroleucon sonchi*	*Pandora neoaphidis*
	creeping thistle	*Uroleucon* spp.?	*Entomophthora planchoniana*
	vining pea	*Acyrthosiphon pisum*	*Pandora neoaphidis*
Stubbings, Herts.	wheat	*Sitobion avenae*	*Pandora neoaphidis* *Entomophthora planchoniana* *Conidiobolus* sp.
	wheat	*Sitobion fragariae*	*Pandora neoaphidis* *Zoophthora phalloides*
	margin grasses	*Sitobion avenae*	*Pandora neoaphidis* *Entomophthora planchoniana*
		Sitobion fragariae	*Pandora neoaphidis* *Entomophthora planchoniana* *Zoophthora radicans*
	bramble	*Amphorophora rubi*	*Pandora neoaphidis*
	nettle	*Microlophium carnosum*	*Pandora neoaphidis* *Entomophthora planchoniana* *Neozygites microlophii* *Zoophthora phalloides* *Conidiobolus* sp.

All aphid identifications done by M.S. Taylor (Rothamsted Research). *Pandora neoaphidis* was also isolated from *Microlophium primulae* feeding on cultivated primrose.

Surveys at Colworth House and Radcot Bridge Farm

Low numbers of aphids were found during surveys in 2000 and 2001 conducted on various field margins at the two farms and very few fungal infections were found (Table 1.3). At Radcot Bridge in 2000, no aphids were found during quadrat counts conducted between early June and end-July on two margins, one dominated by wildflowers (especially ribwort plantain and knapweed) and the other dominated by grasses (mostly Yorkshire fog). In a separate grass margin, the mean density from five sample dates was 14.33 living aphids 0.25 m^{-2} (SE = 8.87) and 0.03 infected aphids 0.25 m^{-2} (SE = 0.03). The overall prevalence of fungal infection was 2.5% (n = 283). In 2001, three different margins were sampled and low numbers were again encountered; 35 live aphids were found during quadrat counts on four dates between mid-June to end-July, but no infected aphids were detected.

At Colworth House in 2000, a mean density of 1.18 living aphids 0.25 m^{-2} (SE = 1.19) was found in a grass margin from four sample dates between early-June and mid-July, but no fungal infections were identified from the 220 aphids encountered during sampling. There was a noticeable amount of parasitism in early August in this margin. A total of 16 parasitised mummies were found, almost exclusively from aphids on Yorkshire fog. In addition, a total of 12 live aphids but none infected were found during transects and quadrat counts in a newly-established flower margin and a 20 m wide grass conservation strip at this site. In 2001, 18 live aphids but none infected were found during sampling of three field margins between mid-June to end-July.

Stubbings field margin, Rothamsted Farm

Plant species

The field margin was largely dominated by white clover and grasses during the four years of this study (Table 1.4). There were several changes in plant species composition during this time, especially the complete absence of wild carrot and yarrow after 2000 and the gradual appearance of chicory. In addition, wood false brome had been included as a shade tolerant grass but the flowers were attacked in 2000 by a smut disease (possibly seed-borne) which appeared to reduce the numbers of flowers, and in subsequent years the species was recognised largely by leaf and ligule morphology. Overall, one grass from 11 species and 13 from 20 wildflower species were not recorded during plant surveys, despite their inclusion in the seed mix (Table 1.4). The failure for these species to establish was probably due to soil, pest (pigeons, rabbits) and site-specific factors. A total of 27 plant species were identified which were not present in the seed mix, presumably by seed having dispersed into the margin or from the soil seed bank. The total numbers of species (= species richness) was lowest for these "weeds" in 2001 (Table 1.5).

The optimal appearance of the margin was in 2001, when Yorkshire fog and crested dogstail were dominant grasses and coincided with the largest aphid densities recorded during the four years of sampling. However, from 2002 onwards there was a deterioration in the quality of field margin plants which was partly illustrated by the presence of stinging nettle and thistles in 2002 and 2003, but also by the large abundance of sorrel flower heads which gave the margin an atypical appearance. This deterioration could have been due to a combination of mowing plants to a very short height (< 15 cm) in autumn 2001 followed by dry conditions in spring 2002, which hindered vegetation regrowth. In addition, the perennial ryegrass strip between the

margin and the woodland also deteriorated, possibly because of a lack of appropriate mowing, and bare patches of soil were visible especially towards the northern portion of the field margin.

Table 1.4: Plant species recorded at Stubbings field margin from a seed mix sown in March 2000. Figures in columns for sample dates indicate presence of individual species in 60 quadrat counts, except for 2000 when 30 counts were taken. Final column is the mean percent recorded over four year, SE in parentheses.

		28/07/00	13/07/01	11/07/02	15/07/03	% presence
GRASSES						
Holcus lanatus	Yorkshire fog	21	60	45	51	82.5 (18.2)
**Brachypodium sylvaticum*	wood false-brome	26	39	24	39	64.2 (64.2)
Festuca rubra	red fescue	29	43		28	53.8 (32.1)
**Poa nemoralis, P. trivialis*	woodland meadow, rough meadow		54	6	9	28.8 (32.1)
Cynosurus cristatus	crested dogstail		52		2	22.5 (32.7)
Agrostis capillaris	common bent	3	22			11.7 (20.8)
Phleum bertolini	small timothy	5		7		7.1 (14.5)
Bromus unioloides	rescue brome	7				5.8 (17.1)
Bromus secalinus	rye brome		6			2.5 (11.2)
Dactylis glomeratus	cocksfoot					0.0
WILDFLOWERS						
Trifolium repens	white clover	28	58	51	42	86.3 (17.2)
Rumex acetosa	sorrel	24	55	49	49	83.8 (11.5)
Daucus carota	wild carrot	28				23.3 (34.2)
Achillea millifolium	yarrow	18				15.0 (27.4)
Leucanthemum vulgare	oxeye daisy	4				3.3 (12.9)
Silene dioica	red campion	1		1	4	2.9 (8.4)
Lotus corniculatus	birdsfoot trefoil		1	1	4	2.5 (8.5)
Alliaria petiolata	hedge garlic					0.0
Anthyllis vulneraria	kidney vetch					0.0
Centauria nigra	knapweed					0.0
Galium verum	ladies bedstraw					0.0
Galium mollugo	hedge bedstraw					0.0
Geum urbanum	wood avens					0.0
Malva moschata	musk mallow					0.0
Medicago sativa	lucerne					0.0
Plantago lanceolata	ribwort plantain					0.0
Prunella vulgaris	selfheal					0.0
Ranunculus acris	buttercup					0.0
Rhinanthus minor	yellow rattle					0.0
Salvia horminoides	wild clary					0.0

* included because of shade tolerance

Seed mix comprised of 80% grasses and 20% wildflowers (by volume). Sowing rate of 200 kg ha^{-1}.

Assistance in plant identifications from P. Lutman, M. Skellern and H. Jones (Rothamsted Research).

Table 1.5: Plant species recorded at Stubbings field margin which were not included in the seed mix sown. Figures in columns for sample dates indicate presence of individual species in 60 quadrat counts, except for 2000 when 30 counts were taken. Final column is the mean percent recorded over four years, SE in parentheses.

		28/07/00	13/07/01	11/07/02	15/07/03	% presence
Sonchus asper	prickly sowthistle	12		17		17.1 (22.5)
Arrhenatherum elatius	false oat grass	15		1		12.9 (24.9)
Atriplex patula	common orache	14				11.7 (24.2)
Cichorium intybus	chicory		10	6	8	10.0 (13.4)
Sonchus oleraceus	smooth sowthistle	7	1		3	7.5 (16.4)
Crepis capillaris	smooth hawk's-beard			12	1	5.4 (15.6)
Stellaria media	chickweed	6				5.0 (15.8)
Cirsium vulgare	spear thistle			3	6	3.8 (10.9)
Galium aparine	cleavers	1		6	1	3.8 (10.5)
Urtica dioica	stinging nettle			4	4	3.3 (9.8)
Raphanus raphanistrum	wild radish	4				3.3 (12.9)
Cirsium arvense	creeping thistle			2	5	2.9 (9.9)
Lapsana communis	nipplewort			1	6	2.9 (10.9)
Geranium dissectum	cut leaved cranesbill	3				2.5 (11.2)
Triticum aestivum	volunteer wheat	3				2.5 (11.2)
Papaver rhoeas	field poppy	2				1.7 (9.1)
Bilderdykia convolvulus	black bindweed	2				1.7 (9.1)
Lolium perenne	perennial rye grass	2				1.7 (9.1)
Matricaria maritima	scentless mayweed	1		1		1.3 (6.3)
Avena fatua	wild oats	1		1		1.3 (6.3)
Polygonum persicaria	persicaria	1				0.8 (6.5)
Anagallis arvensis	scarlet pimpernel	1				0.8 (6.5)
Myosotis arvensis	common forget-me-not				2	0.8 (6.5)
Taraxaxum officinale	dandelion			2		0.8 (6.5)
Trifolium pratense	red clover		1			0.4 (4.6)
Ranunculus repens	creeping buttercup			1		0.4 (4.6)
Veronica officinalis	common speedwell			1		0.4 (4.6)
UNIDENTIFIED				10	6	6.7 (14.3)

Assistance in plant identifications from P. Lutman, M. Skellern and H. Jones (Rothamsted Research).

Aphids and natural enemies

Fungal species infecting aphids were identified from microscope slide preparations (Table 1.3), while parasitoids were reared out from field collected mummies (Table 1.6).

Low densities of living aphids were found in all four years, both in the field margin and in the adjacent wheat crop, while no infected aphids were found in 2003 (Figures 1.2 and 1.3). In the field margin, maximum densities of 16.85 live aphids 0.25 m^{-2} (SE = 3.57) and 1.27 infected aphids 0.25 m^{-2} (SE = 0.68) were recorded in 2001. In the adjacent wheat crop, a maximum density of 1.02 live aphids 5 $tillers^{-1}$ (SE = 0.18) was recorded in 2001, while a maximum of 0.05 infected aphids 5 $tillers^{-1}$ (SE = 0.04) was recorded in 2000.

Table 1.6: Parasitoids identified from Stubbings, Rothamsted Farm, between 2000-2002

Area	Aphid host	Host plant	Parasitoid	
margin	*Sitobion fragariae*	Yorkshire fog	*Dendrocerus* sp.	
			Alloxysta sp.	
			Aphidius rhopalosiphi	+
			Aphelinus sp.	+
			Phaenoglyphis sp.	
	Hyperomyzus lactucae	prickly sowthistle	*Praon* sp.	+
	Megoura viciae	white clover	*Alloxysta victrix**	
	unknown aphid species	Yorkshire fog	*Ephedrus* sp.	+
		crested dogstail	*Aphidius rhopalosiphi*	+
		Poa sp.	*Phaenoglyphis* sp.	
			Alloxysta	
		common persicaria	*macrophadna**	
		white clover	*Asaphes* sp.	
			Aphidius ervi	+
cereal crop	*Metopolophium dirhodum*	wheat	*Alloxysta* sp.	
	Sitobion avenae		*Asaphus vulgaris*	
			Praon sp.	+
			Aphidius rhopalosiphi	+
			Dendrocerus sp.	
	Sitobion fragariae		*Aphidius rhopalosiphi*	+
			Aphidius ervi	+
			Aphidius rhopalosiphi	+
			Alloxysta sp.	
			Phaenoglyphis sp.	
			Praon volucre	+
			Ephedrus plagiator	+

Parasitoids identified by W. Powell (Rothamsted Research) except * by F. van Veen (Imperial College, Silwood Park, Ascot). Crosses indicate if species are primary, rather than secondary, parasitoids.

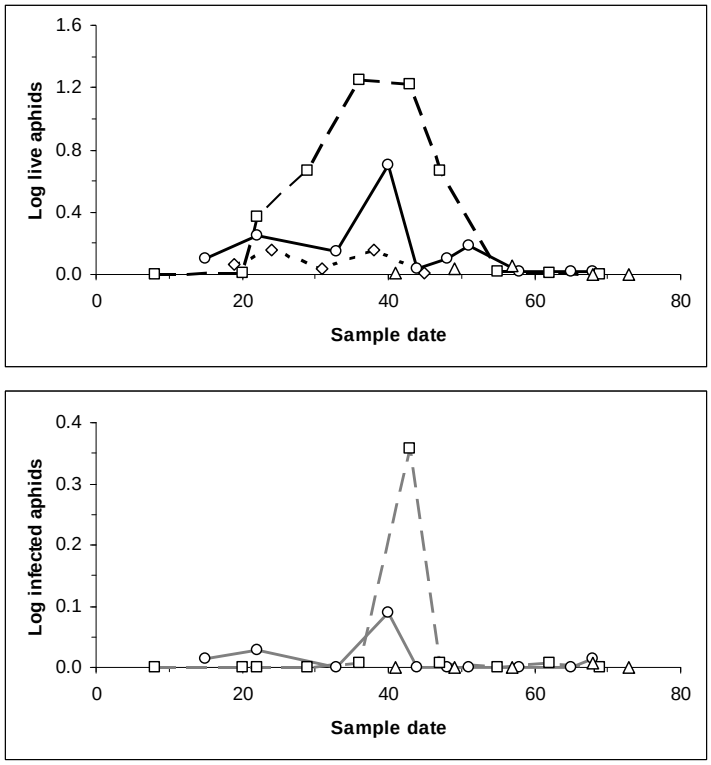

Figure 1.2: Mean densities of live (top) and infected (bottom) aphids at Stubbings field margin in 2000 (○), 2001 (□), 2002 (△) and 2003 (◇). June 1st was Day 1 for all years. Densities for parasitism not shown.

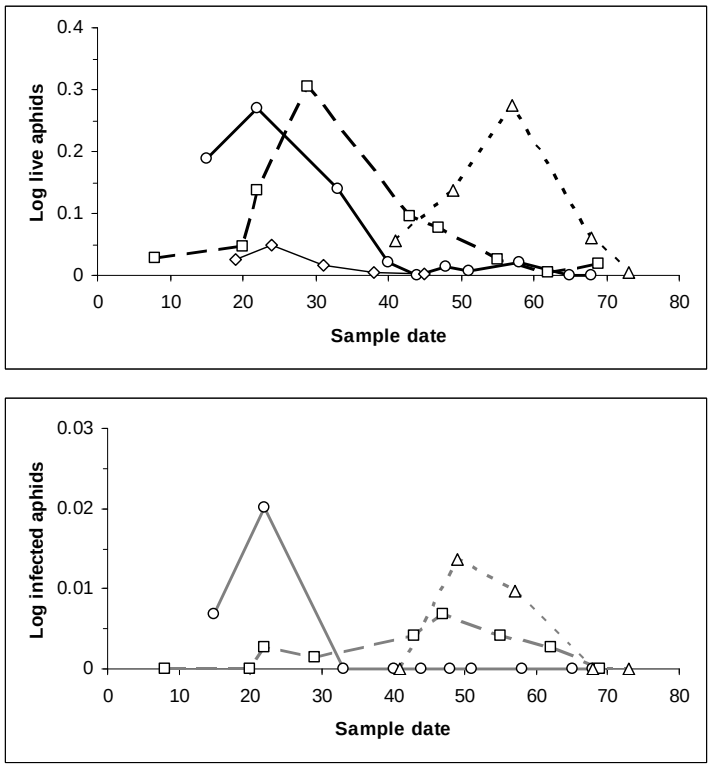

Figure 1.3: Mean densities of live (top) and infected (bottom) aphids in the wheat crop at Stubbings, adjacent to the field margin, in 2000 (○), 2001 (□), 2002 (△) and 2003 (◇). June 1st was Day 1 for all years. Densities for parasitism not shown.

Logistic regression analyses indicated there were no differences between years for prevalences of fungal infection in the field margin ($F_{2, 21} = 1.93$, $P = 0.171$) or wheat crop ($F_{2, 21} = 0.21$, $P = 0.815$). There were also no differences in prevalences of parasitism between years, either in the field margin ($F_{2, 21} = 0.44$, $P = 0.647$) or the wheat crop ($F_{2, 21} = 1.35$, $P = 0.282$) (Figure 1.4).

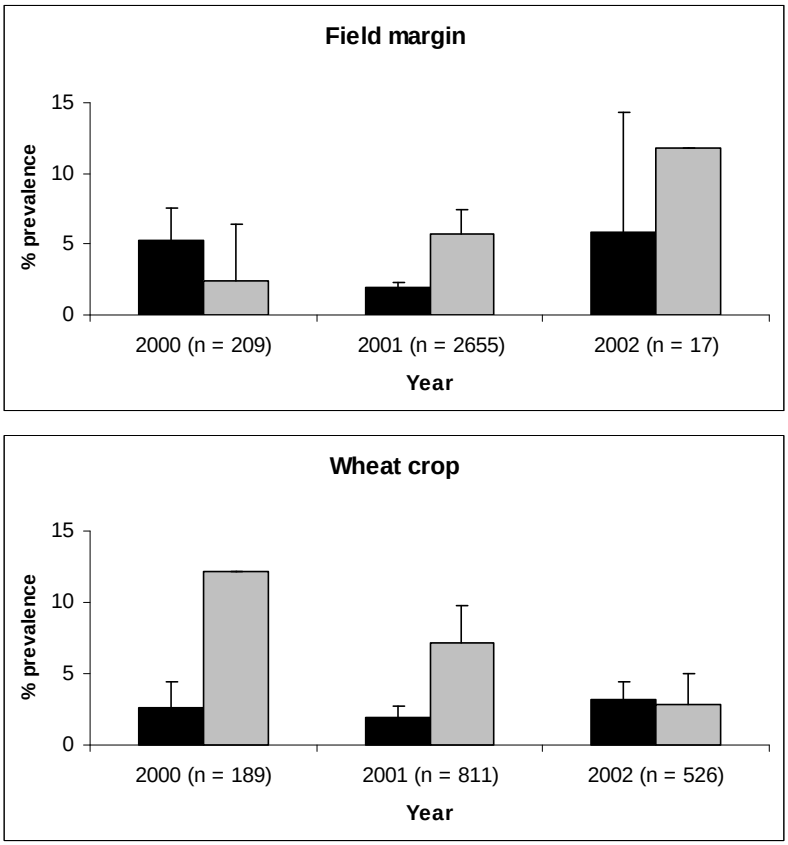

Figure 1.4: Mean prevalences for fungal infection (black bars) and parasitism (grey bars) of aphids from Stubbings field margin and wheat crop. Total numbers of aphids in parentheses. No infection or parasitism recorded in 2003. Bars represent SE, omitted for parasitism in margin in 2002 (SE = 30.06) and in crop in 2000 (SE = 6.94).

Numbers of predators were also recorded in the wheat crop in 2001. The dominant aphid predator was the 7-spot ladybird, *Coccinella septempunctata* L., followed by syrphids, mostly *Episyrphus balteatus*, but spiders (not identified) were not as numerous (Figure 1.5). However, sampling will have underestimated predator numbers since many generalist and specialist predators are nocturnal. It should also be noted that adult ladybirds were found when sampling in the crop throughout August, despite the absence of any aphid hosts.

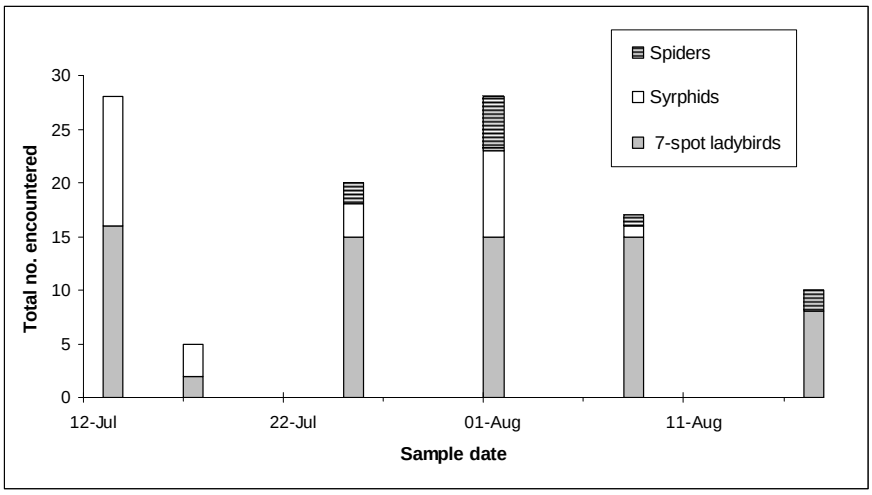

Figure 1.5: Total numbers of predators encountered during sampling of the wheat crop at Stubbings in 2001. On each date, 5 wheat tillers were examined from intersections in a 9 x 7 grid (=315 tillers), with a spacing of 10 m between intersections.

SADIE analyses for counts in 2001 indicated a significant spatial structure (patch, gap) in the field margin for live aphids on 17th July and for infected aphids on 13th July (Table 1.7). SADIE analyses were also conducted to determine whether the spatial distributions of live aphids were associated with aphids showing symptoms of parasitism or infection. Significant associations were found between live and infected aphids on 13th July (Xp = 0.41, P<0.0196), as well as between live and parasitised aphids on 13th (Xp = 0.42, P<0.0196), 17th (Xp = 0.40, P<0.0196) and 25th July (Xp = 0.39, P<0.0196). Furthermore, there was a significant lagged association between parasitism on 6th July and infection on 13th July (Figure 1.6) but not between live aphids and infection or parasitism for any combinations of dates examined (results not shown).

Table 1.7: Summary of SADIE analyses for aphids, fungal infection and parasitism recorded during grid-based sampling at Stubbings field margin in 2001.

					GAP		PATCH	
	date	mean	variance	n	Vj	P	Vi	P
Living aphids	29 June	3.60	112.35	216	-	-	-	-
	6 July	16.85	162.88	1011	-	-	-	-
	13 July	15.22	203.36	913	-2.08	0.0256	-	-
	17 July	3.67	18.40	220	-3.22	0.0000	3.38	0.0000
Infected aphids	6 July	0.017	0.017	1	-	-	-	-
	13 July	0.233	0.284	14	-2.20	0.0128	1.91	0.0256
	17 July	0.017	0.017	1	-	-	-	-
Parasitised aphids	6 July	0.58	0.48	35	-	-	-	-
	13 July	1.70	3.43	102	-	-	-	-
	17 July	0.48	0.37	29	-	-	-	-

Hyphens denote non-significant results or analyses omitted because of small sample sizes.

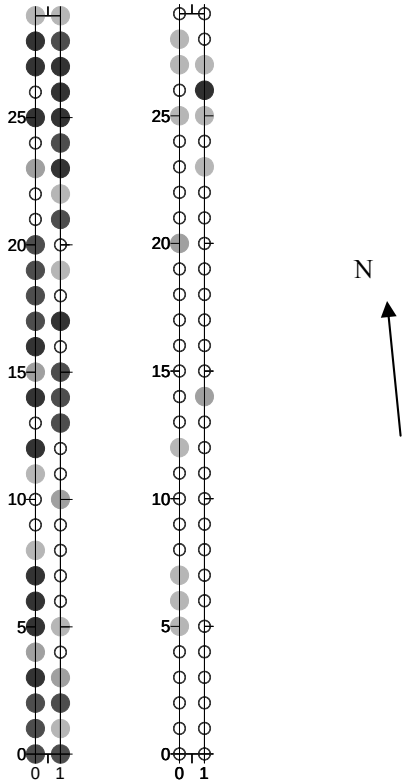

Figure 1.6

Illustration of lagged associations in the spatial distributions of infected and parasitised aphids at Stubbings field margin in 2001. Association (dark grey) or dissociation (light grey) between natural enemies is shown for individual quadrat sample points. The left hand diagram illustrates a significant association between parasitised mummies on 6 July and infected cadavers on 13 July (Xp = 0.45, P<0.0196), while the right hand diagram represents counts of parasitism and infection on 13 July (Xp = 0.12, ns).

Discussion

A total of 11 aphid species were found infected with *P. neoaphidis* and six isolates were added to the culture collection for evaluation in laboratory studies. The numbers of species with infection and successful isolations may seem to be small from four years of field work, but is related to the low aphid densities which were encountered during farm surveys and intensive field sampling at Rothamsted. Two exceptions were the moderate to high numbers (≥ 20 per plant) of *M.carnosum* which were consistently found on nettles from both surveys at different farms and at Rothamsted (see Section 1.4 on nettle aphids), as well as moderate numbers of *S. fragariae* found on grasses in Stubbings field margin during 2001, which provided an opportunity to study spatio-temporal interactions between parasitoids, fungi and their shared aphid hosts.

Overall, 72 aphid species have been listed as hosts of *P. neoaphidis* throughout the World, most of which were pest species feeding on crop plants (Hemmati, 1998). From the current work, *Cavariella* spp. on

hogweed are potentially new records for infection by *P. neoaphidis*. Important sources of *P. neoaphidis* in field margins and hedgerows were *Sitobion* spp. on Yorkshire fog and the nettle aphid, *Microlophium carnosum*, on stinging nettles. Other potentially beneficial plants for *P. neoaphidis* included grasses (crested dogstail, cocksfoot), legumes (e.g. bird's-foot trefoil, clover), as well as teasel, bramble, and hogweed. However, low numbers of aphids were recorded during field studies and many more combinations may have been identified in years with larger aphid densities. Intensive spatio-temporal sampling at Stubbings gave some evidence for an association between infection and parasitism in 2001. This provided some confirmation of laboratory studies which indicate that parasitism causes increased infection because of agitated behaviour by aphids. Limitations in sampling studies included low numbers of aphids and natural enemies from which to obtain detailed information on interactions and the lack of treatment comparisons (boundaries with and without field margins) as well as deficiency in replication. Treatment and replication limitations are considered to be common faults with many studies on agri-environment schemes (Kleijn and Sutherland, 2003).

Future studies

The work presented here provided a first attempt to obtain baseline data on the potential use of field margins as habitats to encourage fungal entomopathogens, with *P. neoaphidis* and aphids providing the basis for a model system. A number of factors need further studies if enhancement of fungal entomopathogens is to be incorporated as a key goal for field margin management. These include the manner in which *P. neoaphidis* overwinters within agroecosystems, and issues on appropriate management of margins to preserve arthropods and fungal entomopathogens (e.g. use of livestock to graze margins, timing and frequency of management interventions). None of the margin management issues are being addressed in any of the current field margin research projects being conducted as far as we are aware.

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1.4 Studies on the role of stinging nettles as reservoirs of *Pandora neoaphidis*.

Abstract

Intensive sampling studies were carried out at two sites containing nettles to determine interactions between *Pandora neoaphidis* and the large nettle aphid, *Microlophium carnosum*. Infection of nettle aphids under an

oak tree was 20-30% in 2000 and 2002. Aphid infection of nettle patches in the perimeter of a cereal field was *ca*. 30% in 2002. At both sites, <10% infection was recorded in 2001 and 2003. Infection was found to be greater within the lower canopy of individual nettle plants in 2000 and 2002. Peak infection by *P. neoaphidis* occurred from mid-June at both sites. Field studies on cross-infection between nettle sources and aphids on nearby crops are required to complement poytunnel studies (Section 1.) and to determine if nettles are beneficial in enhancing biological control by *P. neoaphidis*.

Introduction

Stinging nettle, *Urtica dioica*, is a common farmland plant often occurring by roadsides or within field boundaries. The large nettle aphid, *Microlophium carnosum*, is a holocyclic species normally feeding on stinging nettles from May to July, with smaller numbers noted between September and October each year. Nettles have long been considered to be suitable sources of natural enemies on farmland, which switch to aphid and psyllid hosts on nettles when no suitable pest populations are available (Perrin, 1975). Records of entomophthoralean fungi infecting *M. carnosum* have been given from UK, the Netherlands, Switzerland and Poland (Perrin, 1975; Bałazy, 1993; Hemmati, 1998; Barta and Cagáň, 2003).

The objectives of the current work were to determine spatio-temporal relationships between *M. carnosum* and entomopathogenic fungi at two study sites within a cereal agroecosystem, which could have implications for biological control of pest aphids in the adjacent wheat crop.

Methods and Materials

The first site was an area of nettles located under an oak tree at the entrance to Stubbings field, Rothamsted Farm. Within this area of 40 m^2, 15 nettle plants, approximately 0.8-1.0 m apart, were labelled with paper tags. The same plants were evaluated during regular sampling between May to July each year. Plant tags were removed at the end of each season, so that different nettle plants were sampled each year from 2000 to 2003. The second site consisted of 30 nettle patches around the perimeter of Stubbings field, which was approximately 900 m^2. Each patch was separated by 20 m and marked with a bamboo cane. Sampling was done on five plants chosen non-systematically within each patch on each occasion each year from 2001 to 2003.

For both the oak tree and perimeter sites, counts of *M. carnosum* and natural enemies were based on a method developed by Hemmati (1998). On each plant, the first, third, fifth, eighth and tenth leaves (one from each leaf pair) were chosen, and the numbers of live, infected or parasitised aphids were recorded, along with the aphid stages involved (alate adults, apterous adults, nymphs). Samples of infected and parasitised aphids were taken for identification in the laboratory. Fungal species infecting aphids were identified from microscope slide preparations, while parasitoids were reared out from field collected mummies. Statistical analyses are still being conducted on the data but preliminary analyses on aphid densities, infection and parasitism are presented.

Results

Pandora neoaphidis was the dominant fungal species except in 2003 when nearly all infection was caused by *Neozygites microlophii*. *Entomophthora planchoniana, Zoophthora phalloides* and *Conidiobolus* sp. were recorded in smaller numbers. Parasitism was very limited at both sites but *Aphidius microlophi* was the most commonly encountered primary parasitoid, while *Praon* sp. and a megaspilid hyperparasitoid were much rarer (W. Powell, personal communication). Aphid-specific predatory arthropods such as lacewings and ladybirds were not commonly encountered (data not shown). Polyphagous predators such as spiders are generally nocturnal and their presence will have been underestimated. Interestingly, mirid bugs found feeding on nettle aphids in 2002 and 2003 have been identified as *Calocoris stysi*, and is a facultative predator which also feeds on the fruits of nettle plants (A. Haughton, personal communication).

At the oak tree site, a maximum density of 47.13 (SE = 1.80) live aphids leaf^{-1} plant^{-1} was recorded in June 2001, while a peak density of 6.83 (SE = 1.79) infected cadavers was recorded in June 2002 (Figure 1.7). At the perimeter site, maximum densities were recorded in June 2002 for both live ($\bar{X}$ = 10.22 ± 2.56 leaf^{-1} plant^{-1} patch^{-1}) and infected aphids ($\bar{X}$ = 4.23 ± 1.35 leaf^{-1} plant^{-1} patch^{-1}) (Figure 1.8).

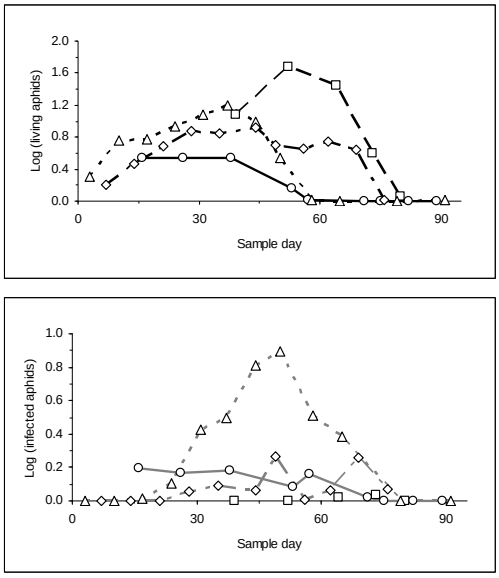

Figure 1.7: Mean densities of live (top) and infected (bottom) nettle aphids at the Stubbings oak tree site in 2000 (○), 2001 (□), 2002 (△) and 2003 (◇). May 1st was Day 1 for all years. Densities for parasitism not shown.

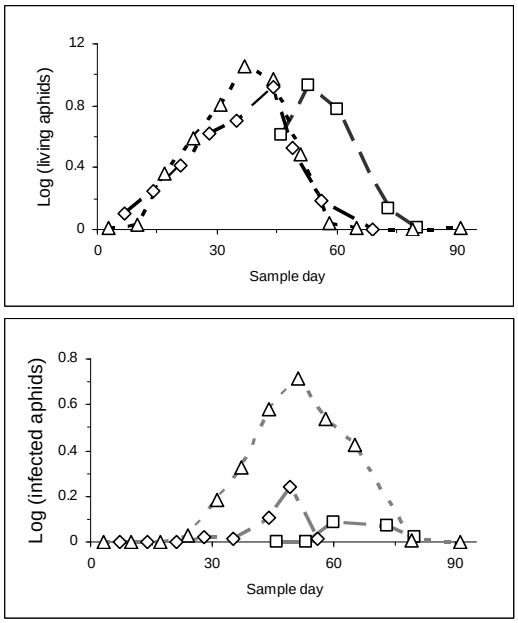

Figure 1.8: Mean densities of live (top) and infected (bottom) nettle aphids at the Stubbings perimeter site in 2001 (□), 2002 (△) and 2003 (◇). May 1st was Day 1 for all years. Densities for parasitism not shown.

Logistic regression analyses indicated differences between years for prevalences of fungal infection at both the oak tree ($F_{3, 31} = 4.17$, $P = 0.014$) and perimeter sites ($F_{2, 22} = 5.42$, $P = 0.012$). There were no differences in prevalences of parasitism between years, either at the oak tree ($F_{3, 31} = 0.42$, $P = 0.742$) or perimeter sites ($F_{2, 22} = 1.18$, $P = 0.326$). Mean prevalence of infection was greatest at both sites in 2001 (Figure 1.9).

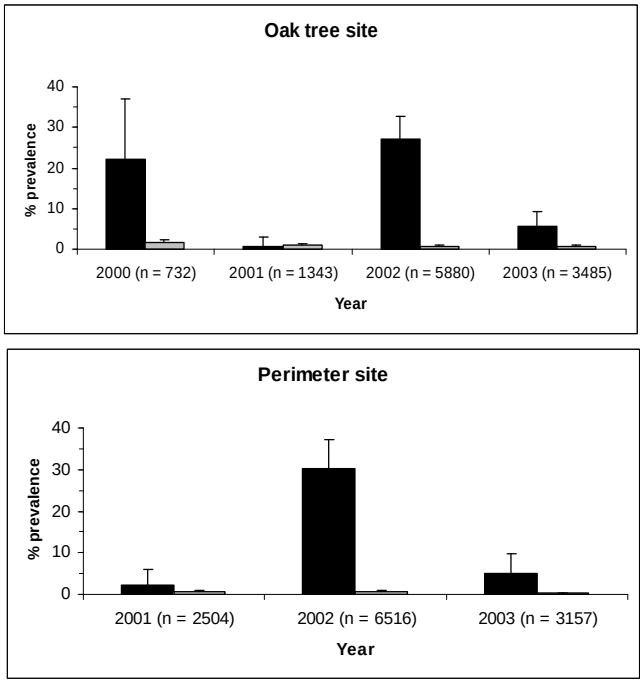

Figure 1.9: Mean prevalences for fungal infection (black bars) and parasitism (grey bars) of aphids from Stubbings oak tree and perimeter nettle sites. Total numbers of aphids recorded in parentheses.

Preliminary analyses indicated that the within-plant distribution of infected cadavers tended to be greater on older leaves in 2000 and 2002 (Figure 1.10), although this may have been confounded by changes in plant height between sample dates.

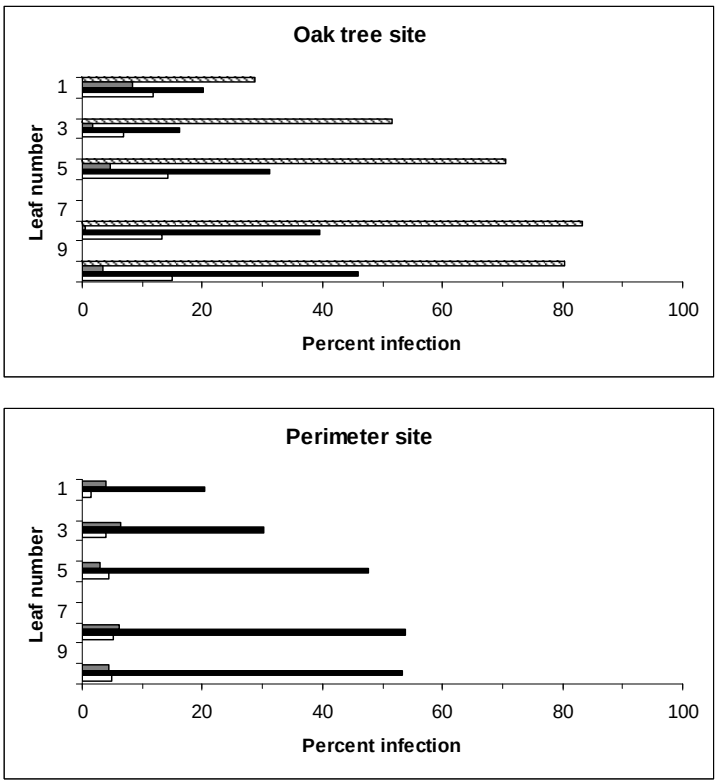

Figure 1.10: Mean prevalences for fungal infection nettle aphids based on individual leaves within nettle plants in 2000 (▨), 2001 (▩), 2002 (■) and 2003 (□). Sampling at Perimeter site initiated in 2001.

Discussion

Between 2000 to 2002, *P. neoaphidis* was the most common entomopathogen but aphid infection in 2003 was dominated by *N. microlophii*, possibly reflecting the adaptation of this species to the exceptionally high temperatures in 2003. Overall, nettle aphid appears to be an important host for a number of entomophthoralean fungi in agro-ecosystems. Peak densities of living aphids occurred during early June at both sites, except in 2001 when it was at the end of June. Peak infection was found from mid-June onwards. Hemmati (1998) carried out studies on nettle aphids in nettles located within the south-eastern hedge boundary of Stubbings field. Moderate infection levels were found in 1995 but not in 1996 or 1997. In 1995, peak infection occurred in mid-July, and highest numbers of infected cadavers were found on leaf numbers 5, 8 and 10, similar to results from the current studies. Reasons for this may include higher humidity in the lower plant canopy, favouring fungal infection, and reduced deactivation of conidia by ultraviolet radiation.

Infection of nettle aphids in June and July may have implications for cross-infection of pest aphids on crops adjacent to nettles. Aphid pests of cereals and beans migrate into crops from May onwards but peak during late-June or July (Roy, 1997; Yeo, 2000). Studies are needed to determine if such cross-infection occurs, enhancing biocontrol by *P. neoaphidis* and other entomophthoralean species

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1.2 Modelling of infection transmission between *Pandora neoaphidis* and the pea aphid

Abstract

A series of pot plant and field experiments were done to estimate parameters for an arthropod - microorganism transmission model involving the pea aphid and *Pandora neoaphidis*. Infection data have been obtained but are still under analysis.

Introduction

Fungal pathogens can be important regulatory factors in the population dynamics of many arthropod species. Aphids are hosts of several entomophthoralean fungi, of which the most common is *Pandora neoaphidis* (Pell *et al*., 2001). Because of its importance in aphid population dynamics, there have been many attempts to utilise *P. neoaphidis* as a biological control agent for aphid pest management (Pell *et al*., 2001). Theoretical models for pathogen transmission and infection could be useful to understand the ecology of *P. neoaphidis* which may have implications for its role in biological control. Specifically, modelling studies should provide information on appropriate host and pathogen densities for successful transmission and persistence in crop and non-crop areas within agroecosystems (Hails, 1997). This would underpin our understanding of disease establishment and spread during epidemics (epizootics). To date, we are only aware of one previous attempt to quantify transmission dynamics between aphids and *P. neoaphidis*, where a four compartment model was used with the cereal aphid *Sitobion avenae* F. (Ardisson 2000; Ardisson *et al*., 1997).

The objectives of the current work were to determine transmission coefficients for *P. neoaphidis* infection in several experimental scenarios.

Materials and Methods

Fungus

Isolate NW 327 of *P. neoaphidis* was used and was originally obtained from an infected *A. pisum* at Rothamsted in the 1970's. It is kept as both *in vivo* and *in vitro* cultures at Rothamsted Research, Harpenden, UK. For experiments, diseased cadavers were produced by sequential transfer.

Aphid and plants

The pea aphid, *Acyrthosiphon pisum*, was used as the test aphid species, feeding on broad bean plants, *Vicia faba* (L.), variety "The Sutton", unless otherwise stated.

Pot plant experiments

1. Decay of infection from diseased cadavers

Aphid cadavers previously infected with *P. neoaphidis* were placed on solidified 1.5% distilled water agar (DWA) in 50 mm diameter plastic Petri dishes. The base of each Petri dish was fixed to a galvanised-wire support positioned next to the stem of a single young broad bean plant. Petri dishes were positioned near the base and apex of each plant, containing equivalent numbers of cadavers. One to two day old apterae of *A. pisum* were confined on individual plants by covering with a muslin gauze sleeve. A total of 12 plants were arranged in a randomised complete block design in a sandpit, with four treatments comprising 0, 10, 20 or 40 cadavers per plant, and there were three replicates per cadaver treatment.

For the experiment, groups of 20 apterous aphids (10-12 days old) were confined within the muslin sleeve of each plant for two hours at sample times 0, 5, 10, 22 and 45 hours after placing infected cadavers onto plants. The experiment was started in the evening to simulate natural patterns of conidia discharge, as conidia are known to be released during late-afternoon and early-evening. After exposure on the plants, the groups of aphids were removed and incubated on fresh broad bean plants enclosed with glass hurricane lamp covers (170 mm length, 60-70 mm internal diam.) in a climate chamber regulated to 18-20 °C,50% r.h. and 18: 6 L: D photoperiod Aphids were checked daily for mortality and infection for five days after recovery.

2. Accumulation of infection

Methodology and procedures were similar to those described above except densities of 0, 20 and 40 cadavers per plant were used. Groups of 20 apterous aphids were exposed on bean plants for periods of 0, 5, 10, or 44 hours. A total of 48 experimental plants (3 cadaver densities, 4 exposure times, 4 replicates of each cadaver-time combination) were arranged in a randomised complete block design in the sandpit area. At each sample time, aphids were removed and transferred to fresh bean plants in a climate chamber (as above) and checked daily for mortality and infection up to five days after retrieval from plants in the sandpit.

Field crop experiment

3. Effect of varying cadaver density

Experiments were conducted in a crop of field beans at Rothamsted. The field (New Zealand) was sown with cultivar "Clipper" and had received standard agronomic inputs. Eighteen bean plants were selected near the edge of the field and Petri dishes (50 mm diameter) containing cadavers on DWA were attached to the undersides of three bean leaves with metal paperclips. Approximately 50 cm of the top of each bean shoot was then enclosed with a muslin sleeve secured with rubber bands at the open ends. Densities of 0, 1, 5, 10, 20, 40 cadavers per plant were used. Groups of 30 aphids were confined within the muslin sleeve of each plant for two hours at sample times 0, 1, 3, 5, 7 and 10 days after placing infected cadavers on bean leaves.

Model fitting of infection data

The probability of being infected as a function of the length of exposure to the pathogen will be modelled in two ways; the "all susceptible" model, derived from earlier models (Anderson and May 1981, Dwyer 1991), and the alternative model incorporating heterogeneity in transmission by allowing a fraction of the aphids to either be "uninfectable", or not be exposed to disease inoculum. This may be due to physiological resistance or to a "probability refuge" where the aphid fails to come into contact with the pathogen (Williams and Hails 1994).

Results and Discussion

Pot plant experiments: The proportion of infection was greatest in aphids exposed five hours after cadavers had been placed on bean plants, rather than at 0, 10, 23 or 45 hours and the largest numbers of cadavers generally provided more infection (Figure 1.11). This indicates there is a brief window when conidia production is maximal for direct infecton of aphids, or for indirect acquistion of conidia by aphids moving on leaf surfaces. The second experiment involved holding aphids with cadavers for varying amounts of time rather than the two hour exposure used in the previous experiment. In this case, infection was greatest at 45 hours compared with 0, 5 or 10 hours (Figure 1.12).

Field crop experiment: Proportion of infection was greatest at 0 and 1 days but declined thereafter. There was an indication that infection was host density-dependent for cadavers sporulating between 0 and 1 day (Figure 1.13).

Model fitting: Parameter estimations for transmission coefficients, refuge size, and threshold densities are in progress. Preliminary results indicate that rate of transmission is not greatly influenced by inoculum density.

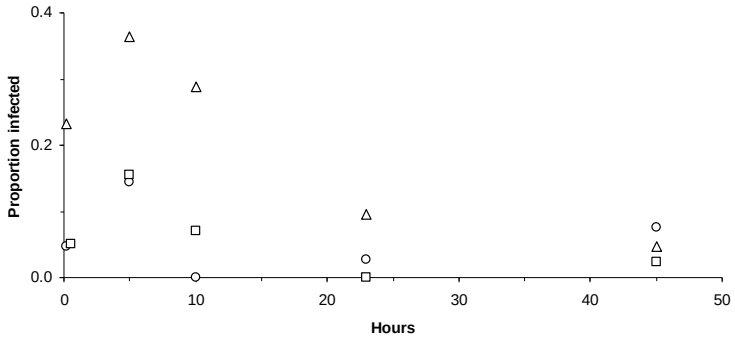

Figure 1.11: Infection of *A. pisum* exposed for two hours to 10 (○), 20 (□) or 40 (△) infected cadavers of *P. neoaphidis* to assess decay of infection in potted bean plants.

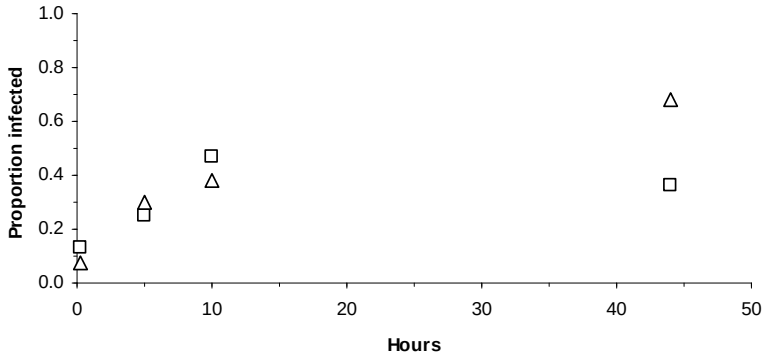

Figure 1.12: Infection of *A. pisum* by 20 (□) or 40 (△) *P. neoaphidis* cadavers to assess increase and infection at different time intervals in potted bean plants.

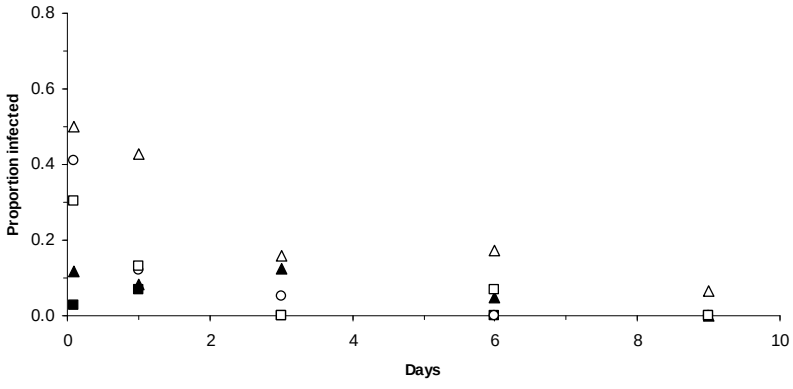

Figure 1.13: Infection of *A. pisum* exposed for two hours to 1 (■), 5(▲), 10 (○), 20 (□) or 40 (△) infected cadavers of *P. neoaphidis* to assess decay of infection in a field bean crop.

Acknowledgements

Experimental design and model parameterisation has been done in collaboration with R.S. Hails (NERC CEH, Oxford). All experiments were done at Rothamsted.

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1.3 Aerial dispersal and infection by *Pandora neoaphidis* conidia in agroecosystems

Abstract

In a polytunnel experiment, conidia of *Pandora neoaphidis* from sporulating nettle aphid cadavers on nettle plants were able to initiate infections in aphid colonies on bean or barley located within 1 m of the nettle source. In a field experiment, conidia dispersal in bean and wheat crops was assessed using rotary samplers. Conidia were mostly caught within 2 m of inoculum sources but could be detected at 12 m away from sources within both crops. Infection of pea and cereal aphids varied between 5-15% within 2 m of inoculum sources. In a separate study, attempts to initiate epizootics in caged bean and wheat crops were unsuccessful probably because of poor fungal inoculum quality.

Introduction

Factors affecting the aerobiology and dispersal characteristics of conidia will greatly influence the development and spread of epizootics by insect-pathogenic fungi. Discharge of *P. neoaphidis* conidia from diseased aphids has a diurnal pattern, starting early in the evening and ceasing by early morning, which coincides with cooler air temperatures and higher relative humidities (Hemmati *et al.*, 2001).

There have been very few studies on the aerial dispersal and infection caused by entomopathogenic fungi. Steinkraus *et al.* (1996) measured concentrations of *Neozygites fresenii* conidia in cotton fields in southern USA, during the development of a prediction system for biological control of the cotton aphid, *Aphis gossypii*. Techniques normally employed for plant pathogens have been used with Entomophthorales because conidia are relatively large for entomopathogens (ca. 20-30 µm width, 30-90 µm length) and have characterisitic morphologies which assist enumeration of conidia under a light microscope.

Two field studies and one polytunnel experiment were carried out in 2002 and 2003, respectively. In the polytunnel experiment, infection of pest aphids downwind of infected nettle aphids on nettle plants was assessed. In the first field study, distances travelled by *P. neoaphidis* conidia in a bean and wheat crop were quantified and related to aphid infection. In the second field experiment, the development of infection by *P. neoaphidis* was investigated in large cages situated within bean and wheat field crops.

Methods and Materials

Aerial dispersal of *P. neoaphidis* from non-pest aphids to pest aphids in a polytunnel

Groups of 50 *Microlophium carnosum* adults, previously inoculated by isolate NW 327, were carefully placed on eight week old nettle plants and covered with transparent polyethylene bags. The plants were then transferred to a controlled environment cabinet to allow for infection to develop. At three days after exposure, plants were carefully removed from the 18°C cabinet and transferred to an unheated polytunnel (2.6 x 5.5 x 22.0 m), and arranged in rows of three on the floor of the tunnel. Three week old bean were then placed adjacent to the infested nettle plants at distances of 0.1, 0.5 and 1.0 m downwind from nettle plants, and three bean plants were placed at each distance. Thereafter, 15 young apterous adult *Acyrthosiphon pisum* were transferred to the bean plants, which were maintained in the polytunnel overnight for a period of 12 h. A similar layout was used for nettles and one week old barley plants containing apterous adult *Metopolophium dirhodum*, so that experiments were conducted simultaneously with the two pest aphid species. Bean and barley control plants were placed 1 metre upwind of nettles containing sporulating aphid cadavers. Numbers of infected aphids on crop plants were analysed using logistic regression (GenStat Committee, 2002). Overall differences between experimental runs were removed before fitting treatment terms and their interactions.

Aerial trapping of conidia and aphid infection in the field

Fungal cultures

Laboratory produced mycelial mats of isolate NW 327 were used as sources of fungal conidia. Mats were produced in a two step process. In the preculture step, ten fungal plugs (each 5 mm diameter) were removed from a culture growing on egg yolk agar and added to 50 ml of milk medium (1% w/v yeast extract, 1.6% w/v glucose, 10% v/v semi-skim milk) in a 250 ml Erlenmeyer flask. After two days growth on a rotary shaker at 200 rpm and 20 °C, 25 ml samples were pipetted into 120 ml of fresh medium in 250 ml Erlenmeyer flasks. Liquid cultures were maintained in this second step for a further six days at 200 rpm annd 20 °C. Fungal mycelium was extracted by filtering the contents of each shake flask through a Buchner funnel with two filter papers (Whatman No. 9). Mycelial mats on filter papers were then placed overnight at

10 °C in plastic sandwich boxes containing moist paper towels to ensure high humidity. This process ensured that formation and discharge of conidia was initiated before mats were placed out for experiments.

Rotorod samplers

Rotorods were used to trap aerial conidia using techniques developed previously at Rothamsted (Hemmati, 1998). Each rotorod comprised of acetate strips attached to brass rods which were mounted on electric motors. The acetate strips were coated with a thin layer of resin to trap conidia. Rotorods were fixed individually to 1.5 m tall steel poles before being placed out in a bean and wheat crop at White Horse II, Rothamsted Farm. Within each crop, rotorods were placed at distances of 1, 2, 4, 6 and 12 m upwind and downwind from the mycelial mats, and another rotorod was placed at 0 m. Mycelial mats were suspended vertically at the height of the crop on the afternoon of August 2nd, when the experiment was started. Rotorods were timed to operate between 15:00 hrs until 08:00 on each of three sample dates. Acetate strips and brass rods were replaced on the mornings of August 3rd and 4th and the experiment was completed on August 5th. After trapping periods, strips were removed from brass rods in the laboratory and mounted in Gelvatol® under coverslips. Conidia on strips were counted under a light microscope at X20 magnification.

Aphid infection

Fungal infection was assessed on August 2nd and 3rd by overnight exposure of live apterae (sentinel aphids) at the 1 and 2 m positions upwind and downwind, as well as at 0 m. Aphids were placed in small Petri dishes (5 cm diameter) containing excised leaves mounted on 1.5% water agar. Lids with gauze covered openings were used to allow entry of fungal conidia but prevent escape by aphids. Petri dishes with aphids were suspended approximately 20 cm below rotorod samplers. In the bean crop, *A. pisum* feeding on broad bean was used, while *M. dirhodum* feeding on barley was used in the wheat crop. After overnight exposures to fungal conidia, aphids were transferred to fresh leaf sections mounted on agar in Petri dishes. Aphids were examined daily for infection or death by other causes until seven days after incubations were started.

Aphid infection in field cages

Fungal cultures

Isolate NW 327 growing on egg yolk agar (Wilding and Brobyn, 1980) was used as a source of *P. neoaphidis* conidia. Fungal plugs (each 5 mm diameter) were cut out and placed on 1.5% distilled water agar overnight at 10 °C to initiate conidia formation and discharge. Groups of five plugs were placed on water agar in Petri dishes (5 cm diameter) before being suspended horizontally at crop height, and a total of 20 plugs were used in one bean or wheat cage.

Aphid infection

Large cages were erected approximately 50 m away from the dispersal experiments being conducted in White Horse II. Each cage (2.74 m length, 2.74 m width, 1.83 m height) consisted of a steel pole framework covered by heavy plastic mesh with access through zipped fasteners. Two cages were placed in the bean and

wheat crop. The downwind cage of each pair was used as the control while fungal mycelium was placed in the centre of each of the upwind cages. In each of these cages, three Petri dishes containing sporulating mycelial mat discs (each 9 cm diameter) were suspended at crop height attached to a bamboo cane by wire supports. Aphids feeding on excised leaves mounted on tap water agar in Petri dishes were suspended at crop height at 0.5, 1.0 and 1.5 m away from the centre on each of the four sides of each cage.

For cages on wheat, mycelial mats and live *M. dirhodum* were left overnight on August 6th, 7th and 8th, while mycelial mats and live *A. pisum* were left out on August 12th, 13 and 14th in the field bean cages. After overnight exposures, aphids were removed and placed on fresh leaf material in clean Petri dishes in the laboratory. Aphids were then monitored daily for infection up to seven days after collection.

Results

Aerial dispersal of *P. neoaphidis* from non-pest aphids to pest aphids in a polytunnel

Infection by *P. neoaphidis* was detected with *A. pisum* on bean and *M. dirhodum* on barley caused by conidia discharged from sporulating cadavers on nettle plants. Infection, however, declined significantly with distance from the nettles for both *A. pisum* ($F = 7.78$; $df = 2, 14$; $P = 0.005$) and *M. dirhodum* ($\chi^2 = 3.24$; $df = 2$; $P = 0.039$). With *M. dirhodum*, there was no infection on barley plants located 0.5 and 1.0 m downwind of nettles. However, for *A. pisum* infection declined approximately 3-fold from 0.1 to 1.0 m (Figure 1.14).

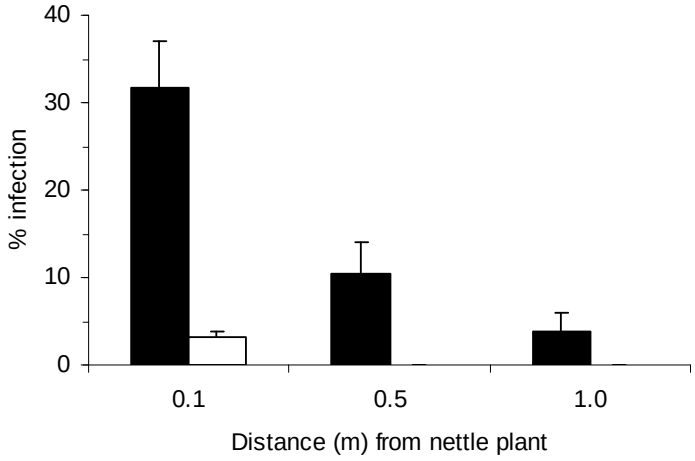

Figure 1.14: Mean infection of *A. pisum* on bean (■) and *M. dirhodum* on barley (□) located at different distances from an augmented source of *P. neoaphidis* infection on nettle. Mean pooled over three replicates and two experiments for each aphid species. Bars denote SE.

Aerial trapping of conidia and aphid infection in the field

Rotorod transects were aligned with the general wind direction for the field site (Figure 1.15). Numbers of conidia trapped in the bean crop were greatest on the second sample day, and numbers were ca. 22-fold greater than on the first or third sample days (Figure 1.16). In the wheat crop, most conidia were trapped on the first day and numbers were ca. 100-fold greater than on the second day. No conidia were trapped on samplers in wheat on the third day. Conidia were mostly found on rotorods within 1 to 2 m of the

inoculum source placed centrally within each crop. Conidia were also detected at the maximum distance (12 m) of transects in both crops.

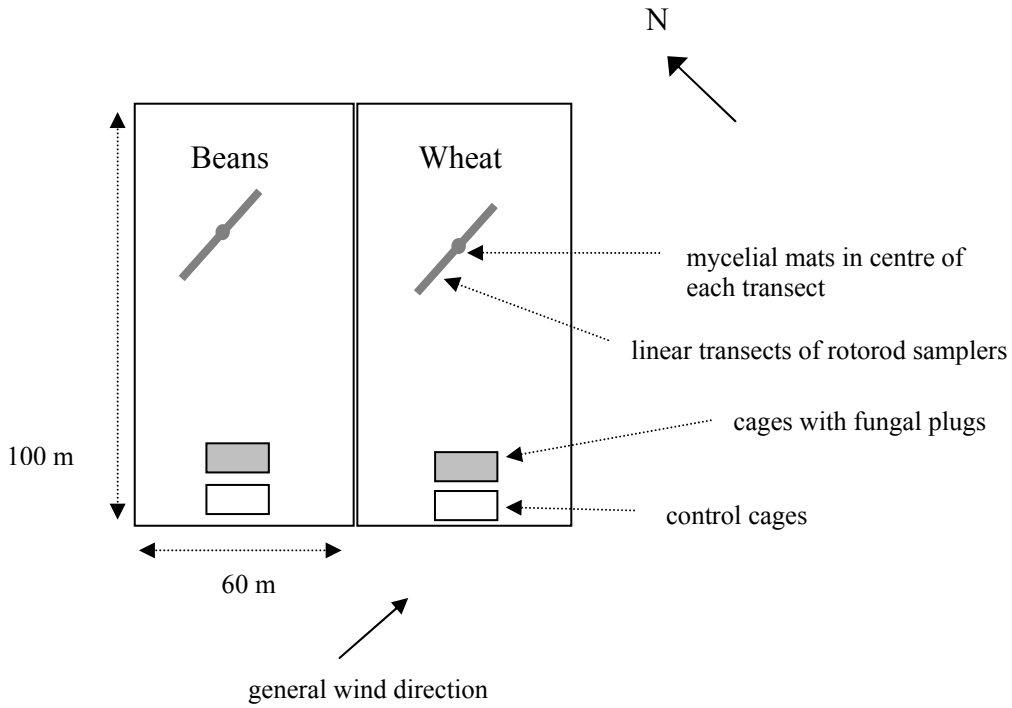

Figure 1.15: Schematic layout of rotorod samplers and cage experiments in contiguous blocks of winter beans and winter wheat at White Horse II in August 2002 (not to scale).

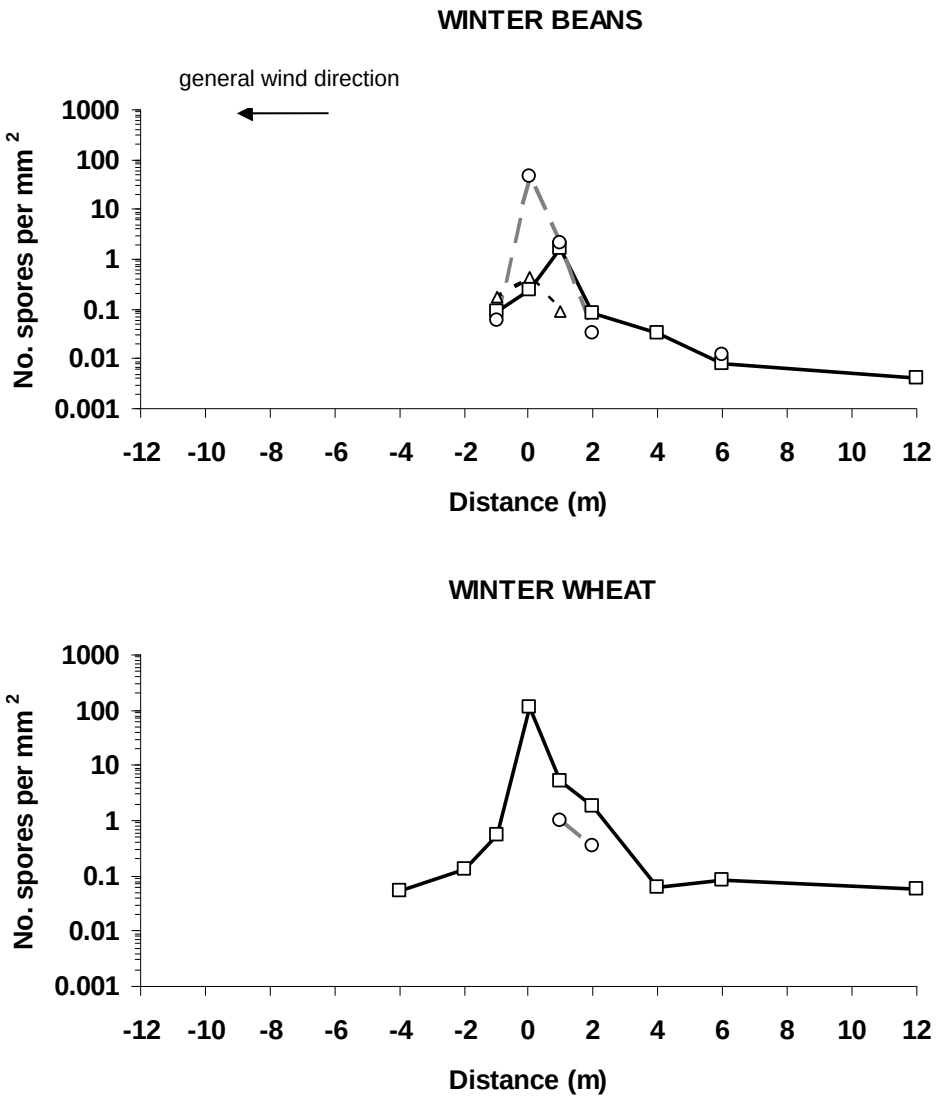

Figure 1.16: Dispersal of *Pandora neoaphidis* conidia from mycelial mats measured using rotarod samplers in a bean and wheat crop on 2-3 August (□), 3-4 August (○) and 4-5 August (△). No conidia were recorded on the last sampling occasion from the wheat crop.

Infection of *A. pisum* varied between 5 and 15% on the two dates tested, but no infection was found for *M. dirhodum* on the first date and was less than 5% on the second date (Figure 1.17). Overall, there appeared to be no relationship between infection in sentinel aphids and conidia numbers trapped on rotorods (results not shown).

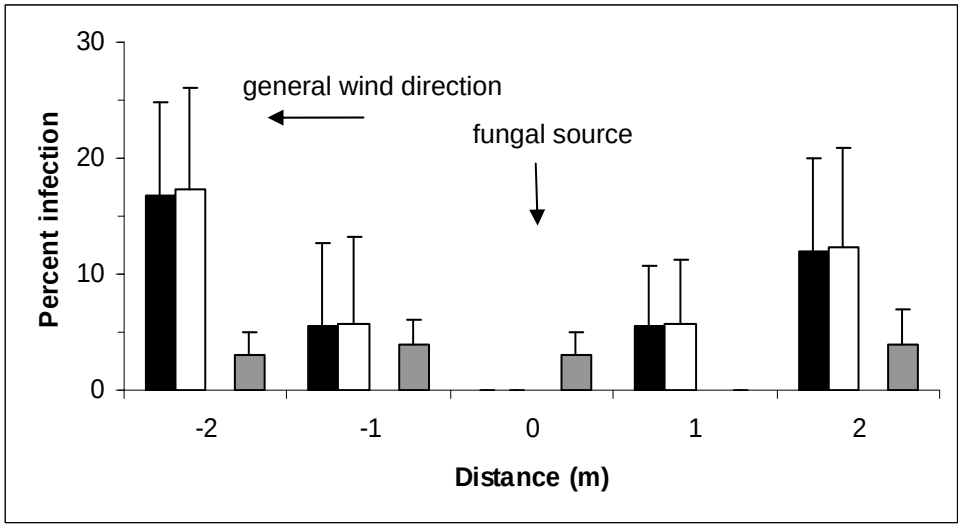

Figure 1.17: Mean infection (+ SE) by *P. neoaphidis* in sentinel aphids placed at different distances from mycelial mats. *Acyrthosiphon pisum* was exposed overnight in a bean crop on 2-3 August (■) and 3-4 August (□), while *M. dirhodum* was exposed in a wheat crop on 2-3 August (no infection) and 3-4 August (▒).

Aphid infection in field cages

With *A. pisum* on field beans, no infections were found in the cage with fungal plugs (n = 521) or in the cage without any fungus (n = 550) from three samples dates. With *M. dirhodum*, infection of 1.3% (n = 228) was found in the fungus treated cage but there was no infection in the control cage (n = 70). All three *M. dirhodum* cadavers had originally been placed at 1.5 m from the fungal source, which was the maximum distance sampled within the cage.

Discussion

Polytunnel experiment: Conidia were shown to disperse from sporulating *M. carnosum* cadavers on nettle plants and cause infection in *A. pisum* on bean and *M. dirhodum* on barley, although infection decreased with increasing distance of the crop plants from the nettles. The large amount of infection in *A. pisum* compared with *M. dirhodum* in this study support results from virulence studies which showed that the former species is more susceptible to infection than the later. Plant architecture may have also played a significant role in the infection process since the broader leaves of the bean plant may have captured more conidia than the leaf blades of barley plants. Aphid infection was only measured after a single night of exposure to cadavers on nettle plants and could be much greater with longer exposure periods resulting in several cycles of infection. Overall, the results demonstrate the potential for conidia dispersal from infected non-pest aphids (*M. carnosum* on nettle) to crop aphids and the potential benefit that may be derived by retaining non-crop plants, such as nettles, in field boundaries.

Aerial dispersal in the field: Hemmati (1998) found *P. neoaphidis* conidia could travel up to 20 m, and probably further, downwind in grassland from a wheat crop during an epizootic in cereal aphids, and most conidia were trapped at 0.8- 1.0 above cereal crops. Furthermore, airborne concentrations of *P. neoaphidis* conidia were usually greatest at night and in the early morning, reflecting the diurnal variation in conidia discharge from aphid cadavers (Hemmati *et al.*, 2001). In the present study, conidia numbers were found to be greatest in the upwnwind rather than downwind transects, suggesting the influence of eddies and gusts within crops on conidia dispersal and impaction. Conidia were captured at the furthest rotorod sampling position (12 m) and were likely, therefore, to have travelled further than this. Site-specific factors, such as neighbouring woods, hedges, field margins or water bodies may also affect conidia dispersal and deposition.

Low amounts of infection were found in sentinel aphids during dispersal studies which may have been due to filtering out of *P. neoaphidis* conidia by gauze barriers used to retain aphids on plant leaves. Future studies may require no barriers on Petri dishes, which may result in smaller numbers of aphids being retrieved for laboratory incubations after overnight field exposures. There were no aphids in bean or wheat crops during experiments, so it was not possible to assess whether the mycelial mats placed in each crop could initiate epizootics, which could have been studied to obtain information such as time taken for *P. neoaphidis* to infect aphid populations at different distances (> 12 m) from inoculum sources. By the third day of the experiment, mycelial mats in bean and wheat crops were very dessicated and covered in contaminating fungi (possibly *Penicillium* spp.). Dessication and contamination are normally observed with mycelial mats of entomophthoralean fungi. The results reported here were for an experiment conducted in August, because no conidia were found on any of the rotorod samplers from mycelial mats during the first attempt to carry out the experiment in July. The inability to consistently mass produce high quality persistent *in vitro* inoculum is a key constraint to the development of *P. neoaphidis* as a bioinsecticide.

Cage experiments: These were not successful as it was not possible to initiate and monitor epizootics; very little infection was found with *M. dirhodum* and no infection with detected with *A. pisum*, which is contrary to other studies and may be an indication of the differences in batches of fungal plugs used in the two cages. Overall, the lack of infection may have been due to the low numbers of conidia discharged from groups of five fungal plugs. Conidia densities were not estimated in cages but copious sporulation was observed around fungal plugs. The inability to initiate epizootics could have been due to a combination of low conidia density and reduced wind eddies inside the nylon mesh covered cages.

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Objective 2: Quantify the impact of aphid physiology and behaviour on susceptibility

2.1 Aphid susceptibility

2.1.1 Pest aphid species

P.A. Shah, S.J. Clark and J.K. Pell (2004). Assessment of aphid host range and isolate variability in *Pandora neoaphidis* (Zygomycetes: Entomophthorales). Biological Control, 29: 90-99.

Abstract

A three tiered assay system was devised for comparing isolates of *Pandora neoaphidis* against seven UK pest aphid species; *Acyrthosiphon pisum*, *Aphis fabae*, *Brevicoryne brassicae*, *Myzus persicae*, *Metopolophium dirhodum*, *Sitobion avenae* and *Rhopalosiphum padi*. In Tier 1, 20 *P. neoaphidis* isolates were screened against all seven species, and 16 fungal isolates gave moderate to high infections of 40-100%. *Acyrthosiphon pisum* was most susceptible whereas *R. padi* was least susceptible to infection. In Tier 2, isolates NW 314, NW 316 and NW 327 were used in concentration-response assays against *A. pisum*, *M. dirhodum* and *M. persicae*. Probit analysis produced median lethal concentrations (LC_{50}) of 8 - 105 conidia mm $^{-2}$ for *A. pisum*, 38 - 92 conidia mm $^{-2}$ for *M. dirhodum* and 105 - 626 mm $^{-2}$ for *M. persicae*. In Tier 3 bioassays, LC_{50} values for the three isolates were tested as discrimatory concentrations against *A. fabae*, *B. brassicae*, *R. padi, S. avenae*, with *M. persicae* as the reference species. Logistic regression analysis indicated that *B. brassicae* and *R. padi* were less susceptible to infection than four other aphid species. The overall ranking of aphids to infection by *P. neoaphidis* was: *A. pisum* > *A. fabae, M. dirhodum, M. persicae, S. avenae* > *B. brassicae, R. padi*. The results suggest that *P. neoaphidis* can cross-infect aphid species which occur both in field crops and non-cropped areas, such as field margins. This is an important feature for the development of conservation biological control strategies.

2.1.2 Non-pest aphid species

Abstract

Virulence of *P. neoaphidis* was assessed in dose-response bioassays against *Microlophium carnosum* on nettle, *Uroleucon jaceae* on knapweed, *Acyrthosiphon pisum* on bean and bird's-foot trefoil, and *Metopolophium dirhodum* on barley and Yorkshire fog. The most susceptible aphid was *A. pisum* feeding on

bean with an LD_{50} of 19 conidia mm^{-2}, while *U. jaceae* had an LD_{50} of 104 conidia mm^{-2} and was most resistant to infection. The other two aphids were intermediate in their susceptibility to infection.

Introduction

Aphids are important crop pests infected by several fungi, of which the most important is *Pandora neoaphidis* (Zygomycetes: Entomophthorales) (Glare and Milner, 1991; Pell *et al*., 2001). Infection can be enhanced by indirect interactions with aphid parasitoids and predators (Fuentes-Contreras *et al*., 1998; Roy *et al*., 2001), but epizootics can often be too late to prevent crop damage. Currently, research studies are evaluating the potential use of arable field margins as habitat refugia to encourage early season infection of pest aphids (Pell *et al*., 2001; Shah *et al*., 2004). The use of conservation biological control requires the provision of favourable conditions within the agroecosystems (e.g. non-cropped areas) to enhance the activity of an entomopathogen (Fuxa, 1998). Pest and non-pest aphids that inhabit non-crop and weed plants within field boundaries may serve as reservoirs for early season multiplication and dispersal of fungal inoculum to crop aphids. The aims of the current studies were to assess the virulence of three *P. neoaphidis* isolates against two non-pest aphids, *Microlophium carnosum* and *Uroleucon jaceae*, and compare this with two pest aphids, *Acyrthosiphon pisum* and *Metopolophium dirhodum*, which were reared on crop and non-crop plants.

Materials and methods

Fungal cultures

Three isolates were used in this study: NW 327, originally isolated from *A. pisum* at Rothamsted, UK, in the 1970's, NW 316, from *M. carnosum* at Rothamsted in 1996, and NW 356, also from *M. carnosum* at Hatfield, UK, in 1999. The isolates were initially grown on Saboraud dextrose agar supplemented with egg yolk and milk in Petri dishes (Wilding and Brobyn, 1980). Cadavers for use in bioassays were produced by sequential transfer from infected to healthy *A. pisum* as described by Wilding (1969). Cadavers were stored in ventilated containers at 4°C until required.

Aphid cultures

All aphid species were maintained in ventilated cages in an insectary at 18°C and 70% RH under a photoperiod of L: D, 18: 6 h. The large nettle aphid, *M. carnosum*, was cultured on nettle, *Urtica dioica*, while *U. jaceae* was cultured on knapweed, *Centaurea nigra.* The pea aphid, *A. pisum*, was originally kept on bean, *Vicia faba* variety "The Sutton", and transferred to bird's-foot trefoil, *Lotus corniculatus*, for experiments. The cereal aphid, *M. dirhodum*, was cultured on barley, *Hordeum vulgare* variety "Gleam", and transferred to Yorkshire fog, *Holcus lanatus*. Both *A. pisum* and *M. dirhodum* were reared on their non-crop host plants for more than four generations before being used in bioassays. All plants were grown in peat-loam soil in a greenhouse at 20°C.

Virulence of* P. neoaphidis *against aphids

The procedure for exposing aphids to *P. neoaphidis* conidia was modified from methodology described by Wilding (1976). Two sets of bioassays were conducted. Firstly, virulence was compared among three isolates against the aphid species and secondly, one isolate (NW 327) was tested across all of the aphid and aphid/plant combinations. Aphid cadavers were removed from storage and four (NW 327) or seven (NW 316 and 356) cadavers were placed in the lid of a 50 mm diam. Petri dish lined with moistened filter paper. The lids were then placed in humid plastic boxes and kept at 10°C to encourage sporulation. After 15 h, the emerging fungus sporulated profusely and was ready for use in bioassays.

Young apterous adult aphids were exposed to conidia in groups of 12 individuals feeding on agar-mounted leaf discs in each 50 mm diam. Petri dish. A 13 mm diam. glass cover slip was placed alongside leaf discs to estimate conidia dose. Aphids were exposed to a range of doses from cadavers by exposing them for periods of 15, 30, 60, 90 and 120 min. Control aphids were maintained under a similar experimental set up but without conidia showers. After showering, groups of aphids were carefully transferred to clean plants. Individual plants were then covered with a lamp glass sealed with plastic film to ensure high RH and incubated at 18°C and L: D 16: 8 h for 24 h. After 24 h, the plastic film was replaced with fine mesh.

The dose of conidia received by each group of aphids was estimated by fixing the coverslips placed in the inoculation chamber with 10% cotton blue in lactophenol, on a glass slide. Stained conidia were counted from ten fields of view using a light microscope at x125 magnification with a calibrated 1 mm^2 eyepiece graticule. The numbers of live aphids, aphids infected with *P. neoaphidis* and aphids dead from unknown causes were recorded daily for a period of seven days.

The data from virulence assays were analysed using the probit analysis facilities of the statistical package GenStat (GenStat Committee, 2002).

Results

Differences in virulence among fungal isolates

In general, smaller LD_{50} values were obtained with isolate NW 327 compared with NW 316 or NW 356, indicating NW 327 was more virulent. LD_{50} values for the three isolates were also smallest with *A. pisum* feeding on bean and largest for *U. jaceae*. There also appeared to be a transfer effect for *A. pisum* and *M. dirhodum*, where LD_{50} values were smaller, indicating greater susceptibility, when these two species were feeding on crop rather than non-crop plants (Table 2.1).

Table 2.1: Abbreviated probit results for virulence of three *Pandora neoaphidis* isolates against pest and non-pest aphids with best fit probit model parameters. LD_{50} (conidia mm^{-2}) ± 95% CI.

Aphid (Host plant)	Isolate	LD_{50} (95% CI)
Microlophium carnosum	NW 327	54.3 (44.0-67.3)
(Nettle)	NW 316	123.6 (97.0-166.4)
	NW 356	71.9 (58.2-90.5)
Uroleucon jaceae	NW 327	95.1 (79.2-119.0)
(Knapweed)	NW 316	130.4 (105.5-172.6)
	NW 356	163.1 (117.3-199.9)
Acyrthosiphon pisum	NW 327	13.0 (4.0-20.5)
(Broad bean)	NW 316	49.5 (40.9-60.3)
	NW 356	48.7 (41.2-57.2)
Acyrthosiphon pisum	NW 327	36.4 (24.6-47.6)
(Bird's-foot trefoil)	NW 316	62.3 (56.1-68.5)
	NW 356	68.8 (61.6-75.1)
Metopolophium dirhodum	NW 327	37.7 (31.8-43.5)
(Barley)	NW 316	69.4 (63.3-75.5)
	NW 356	64.0 (52.9-78.4)
Metopolophium dirhodum	NW 327	75.0 (67.7-81.6)
(Yorkshire fog)	NW 316	95.0 (72.8-147.4)
	NW 356	85.3 (68.3-115.5)

Differences in susceptibility amongst aphid species with isolate NW 327

The LD_{50} for *A. pisum* on bean was 2.2-5.7 times smaller than for other combinations, and *U. jaceae* was most resistant to NW 327. LD_{50} values were also larger for *A. pisum* and *M. dirhodum* on non-crop rather than crop plants, confirming results from the first bioassay (Table 2.2).

Table 2.2: Abbreviated probit results for comparison in virulence of *Pandora neoaphidis* isolate NW 327 against pest and non-pest aphids. LD_{50} (conidia mm^{-2}) ± 95% CI.

Aphid (Host plant)	LD_{50} (95% CI)
Microlophium carnosum (Nettle)	40.0 (33.1-46.6)
Uroleucon jaceae (Knapweed)	103.6 (86.4-136.4)
Acyrthosiphon pisum (Bean)	18.2 (12.1-23.3)
Acyrthosiphon pisum (Bird's-foot trefoil)	43.8 (33.6-54.5)
Metopolophium dirhodum (Barley)	66.5 (60.0-72.7)
Metopolophium dirhodum (Yorkshire fog)	74.7 (68.6-80.3)

Discussion

Dose-response bioassays showed differences amongst aphid species and amongst *P. neoaphidis* isolates. An overall ranking of aphid susceptibility based on LD_{50} results was *A. pisum* > *M. carnosum* > *M. dirhodum* > *U. jaceae*. The LD_{50} values of 18-176 conidia mm^{-2} reported here for *A. pisum* and *M. dirhodum* are similar to previously published results. However, there are no previous reports of LD_{50} for *M. carnosum* or *U. jaceae*. Wilding (1976) found LD_{50} values of 11-116 conidia mm^{-2} in consecutive bioassays with one isolate of *P. neoaphidis* tested against *A. pisum*, while LD_{50} values of 16-51 conidia mm^{-2} were determined with *A. pisum* on bean and *M. dirhodum* on barley for isolates NW 316 and NW 327 (Shah *et al*., 2004).

Of the two non-pest aphids, *M. carnosum* was more susceptible to *P. neoaphidis* than *U. jaceae.* Several species of entomophthoralean fungi, including *P. neoaphidis*, are known to infect *M. carnosum* in agroecosystems (Mietkiewski and van der Geest, 1985; Keller, 1991, Hemmati, 1998; Barta and Cagáň, 2003), but there appear to be no equivalent reports for *U. jaceae*. Our results, however, show that this aphid species can be infected by different isolates of *P. neoaphidis* in laboratory bioassays, and the lack of reported

infection under field conditions could point to disparities in physiological and ecological susceptibility (Hajek *et al*., 1996; Nielsen *et al*., 2001).

In conclusion, the laboratory experiments reported here provide baseline data for *P. neoaphidis* infection of pest and non-pest aphids which may be found in non-crop areas, such as field margins, and in adjacent field crops. The most susceptible aphids such as *A. pisum* and *M. carnosum* would be good reservoirs of *P. neoaphidis* while *M. dirhodum* may also be useful, while *U. jaceae* might be a poor host even though it has been shown to be susceptible to *P. neoaphidis*.

Acknowledgements

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2.2 Plant and cultivar influence

Abstract

Mean percentages of aphids infeced by *Pandora neoaphidis* was greater on the original host plant species compared with the alternate host plant species, but the differences were not statistically significant. Infection also varied among aphid species, from *ca*. 20% for *Acyrthosiphon pisum*, 12% for *Metopolophium dirhodum*, and 5% for *Myzus persicae.* In plant variety bioassays, there were also no statistical differences in percentage infection for aphids on original or alternate plant varieties. Mean infections were *ca*. 90% for *A. pisum*, 14% for *M. dirhodum* and 50% for *M. persicae*. The results indicate no overall effects of alternate host plant species or varieties on infection by *P. neoaphidis*.

Introduction

Studies of tritrophic interactions aim to understand the often complex relationships between plants, herbivores and natural enemies. Plants may positively or negatively affect performance of herbivore natural enemies due to their chemistry, the resources they provide and/ or their morphology (e.g. trichomes) (Kareiva and Sahaklan 1990; Agrawal *et al*. 2000). Aphids are a widespread and diverse group of plant herbivores. Adaptation by many aphid species to marked seasonality and ephemeral summer host resources includes polyphenism (sexual, asexual forms) and telescoping of generations in parthenogenic summer females (Dixon 1998). The effects of various predators, parasitoids and fungal pathogens on aphids have been documented in laboratory and field studies. *Pandora neoaphidis* infects aphids from many different genera and it can be an important regulatory factor limiting aphid populations (Pell *et al*. 2001). There have been few studies on interactions between plant species, aphids and *P. neoaphidis*, except for investigations with wheat varieties and the cereal aphid *Sitobion avenae* (Fuentes-Contreras *et al*. 1998; Fuentes-Contreras

and Niemeyer 2000), and intraspecific variation in the pea aphid, *Acyrthosiphon pisum*, to fungal infection from maternal lines initially collected from different non-crop plant species (Ferrari *et al*. 2001; Ferrari and Godfray 2003).

The objectives of the work reported here were to determine if host plant or variety affected infection by *P. neoaphidis* of *A. pisum* feeding on different crop plants and whether possible differences also occurred with the rose-grain aphid, *Metopolophium dirhodum* Walker, and the peach potato aphid, *Myzus persicae* Sulzer.

Materials and Methods

Insects and plants

All aphids were maintained on appropriate host plant species or varieties in ventilated cages at 18 ºC and 70% RH with a 16:8 hour light:dark (L:D) regime. All plants were grown in a peat - loam mix (pH 5.5-6.0; Petersfield Products, Leicester, England) in a greenhouse at 20 ºC.

For host plant species bioassays with *A. pisum* comparisons were made between the standard host plant in our system, broad bean (*Vicia faba* (L.), variety "The Sutton"), and the alternate pea plant (*Pisum sativum* L., variety "Kelvedon Wonder"). With *M. dirhodum* comparisons were made between the standard barley plant (*Hordeum vulgare* L., variety "Gleam") and the alternate wheat plant (*Triticum aestivum* L., variety "Axona"), and with *M. persicae* comparisons involved the standard Chinese cabbage plant (*Brassica campestris* L. subsp. *pekinensis*, variety "Wongbok") and the alternate potato food plant (*Solanum tuberosum* L., variety "Desiree").

For variety bioassays, the standard broad bean variety "The Sutton" was compared with the alternate variety of "Victor" for *A. pisum*; the standard barley variety "Gleam" was compared with the alternate variety of "Regina" for *M. dirhodum*, and the standard Chinese cabbage variety "Wongbok" was compared with variety "Kasumi" for *M. persicae.* Aphids were reared on different host plants or varieties for one month, which was considered sufficient to dissipate maternal and grand-maternal effects due to host plant origin and ensure physiological adaptation to the test host (Ferrari and Godfray 2003).

Pandora neoaphidis cultures

Pandora neoaphidis isolate NW 343 was used in bioassays, and was obtained from infected *M. persicae* at Rothamsted Farm, Hertfordshire, UK, in 1998. The isolate was preserved in liquid nitrogen until required, and cultured on Sabouraud-dextrose agar supplemented with egg yolk and milk (Wilding and Brobyn 1980). The Petri dish cultures were maintained in darkness at 18-20 °C inside humid plastic boxes for 15-20 days prior to use.

Inoculation of aphids with fungal conidia

Methods for inoculating aphids with fungal spores rely on exposure of test insects to "showers" of discharged conidia (Papierok and Hajek 1997). For bioassays, 2-3 day old apterous adults were sorted into batches of 30 individuals and each batch was placed inside a separate inoculation chamber (25 mm length,

21 mm internal diameter). Each batch of aphids was exposed to simultaneous conidia showers from three excised fungal plugs in each chamber for a defined time period. A 13 mm diameter glass cover slip was placed in the centre of each inoculation chamber in order to estimate the conidia dose received by aphids. In order to assess mortality in the absence of fungus, uninoculated, or control, aphids were first kept in glass tubes until inoculations had finished.

After inoculations, inoculated and control aphids were transferred to the appropriate host plant species or variety for incubation. Plants were kept under high humidity (>90% RH) for 24 hours to ensure conidia germination. After 24 hours, any dead aphids were removed and excluded from data analysis. Plants and inoculated aphids were maintained in a controlled environment room maintained at 18 ºC with a 15.5:8.5 L:D photoperiod. Conidia dose within each inoculation chamber was estimated from counts of coverslips viewed under a light microscope at X125 magnification.

For host plant species bioassays, the time duration for fungal inoculation was 0.5 hr, but a 3 hr fungal exposure period was used in variety assays. Each host plant species or variety test was repeated on three different occasions with different batches of plants, aphids and *P. neoaphidis* Petri dish cultures. The numbers of aphids infected was assessed seven days after fungal exposure.

Data for inoculated (numbers infected) and control (numbers dead) aphids were analysed separately using logistic regression. All statistical analyses were done using the GenStat statistical package (GenStat Committee 2002).

Results

Host plant species and infection of aphids by *P. neoaphidis*

There was some evidence for differences in infection between host plants (χ^2 = 3.56; df = 1; P = 0.059), but the probability for significance was slightly greater than the conventional threshold of 5%. Averaged over all bioassay runs with the three aphid species, and ignoring dose, infection was 15.2% (SE = 3.19) on original host plants, which was 1.5 times greater than the mean infection of 10.0% (SE = 2.60) on alternate host plants. For all three aphid species, *P. neoaphidis* performed better (gave greater infection) on original host plants compared with alternate host plants, and least infection occurred for *M. persicae* on its alternate host plant (Figure 2.1).

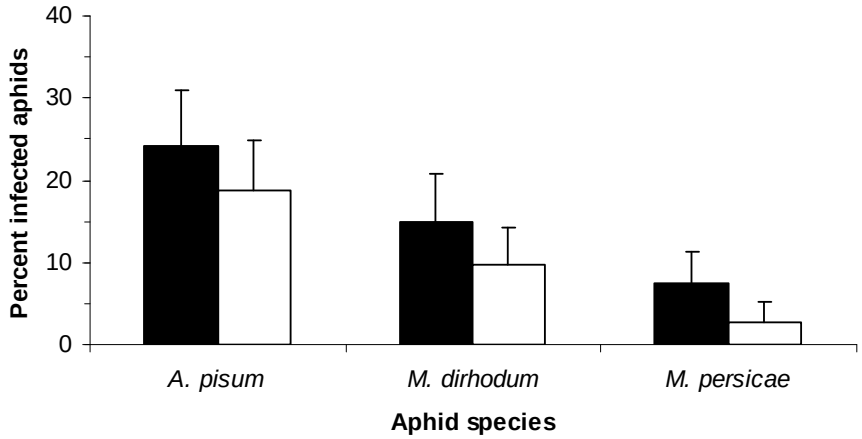

Figure 2.1: Mean infection (+SE) by *P. neoaphidis* averaged over three bioassay runs for each of three aphid species on original (black bars) or alternate (white bars) plant species.

Plant varieties and aphid infection by *P. neoaphidis*

Separate analyses for aphid species did not show any detectable differences in infection between original or alternate varieties with *A. pisum* ($\chi^2 = 2.71$; df = 1; P = 0.100), *M. dirhodum* ($\chi^2 = 0.02$; df = 1; P = 0.898) or *M. persicae* (F = 0.89; df = 1, 6; P = 0.381). Overall, the greatest infection was found with *A. pisum* and least with *M. dirhodum* (Figure 2.2). For all three aphid species, there was no evidence of either a dose-infection relationship or a dose-variety interaction (results not shown).

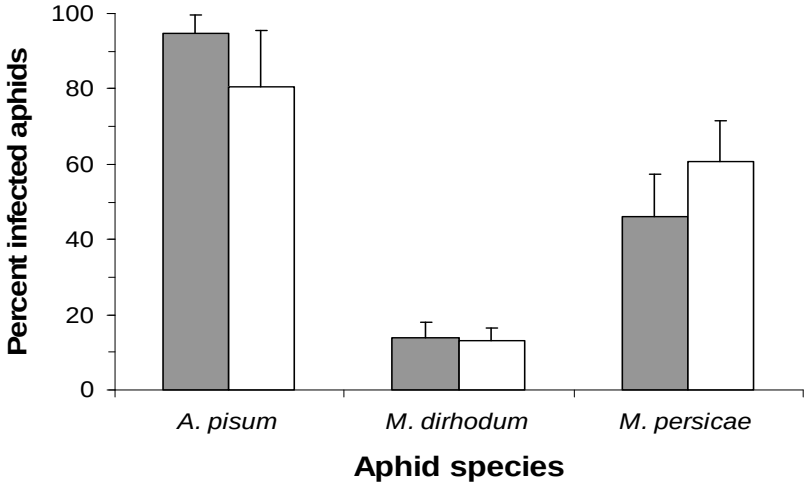

Figure 2.2: Mean infection (+SE) by *P. neoaphidis* averaged over three bioassay runs for each of three aphid species on original (grey bars) or alternate (white bars) plant varieties.

Discussion

Overall, there was a non-significant trend for infection to be greater with aphids feeding on plant species to which they had become adapted during long-term culturing. There were no differences in infection between original and alternate varieties for the three aphid species, which agrees with findings on the lack of difference for *P. neoaphidis* infection of *S. avenae* feeding on susceptible or moderately resistant wheat varieties (Fuentes-Contreras *et al.* 1998; Fuentes-Contreras and Nieymer 2000). All of these bioassays indicated that there were no significant effect of host plant species or variety on *P. neoaphidis* performance. Hence, transmission of *P. neoaphidis* between aphids in crop and non-crop areas is unlikely to be affected by aphid food plant. As no differences in susceptibility related to host plant were found, these experiments were not followed up with behavioural studies on conidia acquisition.

Different clones of *A. pisum* collected from a range of non-crop host plants including *Vicia* spp. and *P. sativum,* as well as large bird's-foot trefoil (*Lotus uliginosus*) and red clover (*Trifolium pratense*) were found to differ in their susceptibility to *P. neoaphidis*, with clones from red clover being most resistant to infection (Ferrari *et al.*, 2001; Ferrari and Godfray, 2003). With pea aphid, it seems likely that there are susceptible and resistant clones feeding on crop and non-crop plants in agroecosystems, along with a range of virulent and less virulent *P. neoaphidis* isolates. The overall impact of all potential interactions is not known but trefoils are probably hosts of the *A. pisum* clones which are highly susceptible to *P. neoaphidis*. Other aphid species also exist as different clonal lines but have been considerably less studied than *A. pisum*.

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2.2.1 Transgenic cultivar influence

Abstract

Studies were done to determine whether susceptibility of the cereal aphid *Metopolophium dirhodum* to the fungus *Pandora neoaphidis* was affected by wheat expressing snowdrop lectin (GNA). The percentages of aphids becoming infected did not differ significantly between the transgenic GNA and non-transformed lines (91% and 82%, respectively). Fecundity did not differ between control and *P. neoaphidis*-treated aphids, and was *ca*. 18 nymphs aptera^{-1}. Time to aphid death caused by *P. neoaphidis* was *ca*. five days for both varieties in two of three assays. Our results indicate that wheat expressing GNA would not compromise the efficacy of *P. neoaphidis* as a biocontrol agent.

Introduction

Transgenic plants have potential for improving agricultural production, by directly increasing crop yields or improving nutritional value of the harvested produce. Indirect advantages of transgenic plants may involve a reduction of synthetic inputs (e.g. pesticides), benefiting biodiversity and wildlife conservation (Hunter, 2000). Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) can confer partial resistance against aphids and other sucking pests by reducing fecundity and longevity (Down *et al.*, 1996; Stoger *et al.*, 1999). Various forms of risk assessment are necessary to evaluate possible ecological impacts of genetically modified plants in agroecosystems. Possible indirect effects of GNA plants against aphid natural enemies have been carried out with predators and parasitoids (e.g. Birch *et al.*, 1999), but there have been no previous studies involving entomopathogenic fungi, which are also important components of aphid natural enemy guilds in agroecosystems (Pell *et al.*, 2001). The most common species infecting aphids in temperate agroecosystems is *Pandora neoaphidis* (Zygomycota: Entomophthorales). This fungus infects adults and nymphs of many genera of aphids but not other arthropods (Pell *et al.*, 2001).

The objective of the current work was to determine whether infection of the rose-grain aphid, *Metopolophium dirhodum*, by *P. neoaphidis* was affected by aphids feeding on transgenic GNA expressing wheat compared to the non-transformed parental line.

Materials and Methods

Transgenic wheat

Co-transformation of wheat (*Triticum aestivum* variety "Bobwhite") was done by particle bombardment of immature embryos (Vasil *et al.*, 1992). The plasmid construct containing the GNA coding sequence was co-bombarded with a plasmid containing the *bar* gene for herbicide resistance fused to the

constitutive promoter ubiquitin 1. Segregation of transformants was carried out on the basis of herbicide resistance. The experimental line used in the study was designated GNA150 and transgene expression levels were determined by immunoassay using Western blot analysis (Stoger *et al.* 1999).

Insects and plants

Aphids were pre-adapted to transgenic or non-transformed wheat lines for one month (*ca*. four generations) before use in bioassays. All aphids were maintained in ventilated cages at 18 ºC and 70% RH with a 16:8 hour light:dark (L:D) regime. Wheat plants were grown in a peat - loam mix at 20 ºC for seven days before being used in bioassays.

***Pandora neoaphidis* cultures**

Isolate NW 343 (Rothamsted culture collection) of *P. neoaphidis* was originally obtained from infected *Myzus persicae* at Rothamsted Farm, Hertfordshire, U.K., in 1998. The isolate was cultured on Sabouraud-dextrose agar supplemented with egg yolk and milk (Wilding and Brobyn 1980), and Petri dish cultures were maintained at 18-20 °C for 15-20 days before use.

Treatment of aphids with fungal conidia

Methods for inoculating aphids with fungal conidia rely on exposure of test insects to "showers" of discharged conidia (Wilding, 1976). For bioassays, 10-12 day old apterous adults were randomly divided into four batches of 30 individuals and each batch was placed inside separate open-ended glass cylinders (25 mm length, 21 mm internal diameter). Aphids were exposed for 3 h to conidia showers from three excised fungal plugs in each cylinder. A 13 mm diameter glass coverslip was placed in the centre of each cylinder, and the conidia dose received by aphids was estimated from 20 counts when coverslips were viewed under a light microscope at X125 magnification. Untreated aphids were kept for 3 h without any fungal plugs.

After conidia showering, individual aphids were transferred to single seedlings and maintained in a controlled environment room at 18 ºC with a 15.5:8.5 L:D photoperiod. Aphids were assessed daily for nymphal production and death. The bioassays were repeated on three separate occasions. Statistical analyses involved logistic regression for data on mortality (death by *P. neoaphidis* and from other causes) and infection (death by *P. neoaphidis* only), comparisons of nymphal production using a log-linear model (GLM with Poisson error and log link), and Weibull distributions for survival analyses were fitted to infection data. All statistical analyses were done using the GenStat statistical package (GenStat Committee, 2002).

Results

Plant lines and aphid infection by *P. neoaphidis*

Immunoassay of the transgenic wheat line (GNA150) by Western blot analysis demonstrated that the transgene product (GNA) was processed correctly and that expression levels in tissues sampled were approximately 0.1% total soluble protein and expression was greatest in younger tissues (data not shown).

The uninoculated groups of aphids showed no difference in mortality between the two lines (χ^2 = 0.47; df = 1; P = 0.493), and means were 9.7% (SE = 3.49) and 13.4% (SE = 4.17) for the GNA and parental lines, respectively. For fungus-treated aphids, there was also no difference in mortality between the wheat lines (χ^2 = 2.10; df = 1; P = 0.148), with means of 93.9% (SE = 2.94) and 85.3% (SE = 4.30) for GNA and parental lines, respectively. Infection by *P. neoaphidis* did not differ between bioassay runs (F = 0.38; df = 2, 3; P = 0.71). Analyses omitting this term showed no relationship between conidia dose and infection (F = 1.03; df = 1, 2; P =0.416), no plant line effect (F = 1.21; df = 1, 2; P = 0.385) and no interaction between dose and plant line (F = 4.80; df = 1, 2; P = 0.160). Means for *P. neoaphidis* infection were 90.9% (SE = 3.54) and 82.4% (SE = 4.62) for GNA and parental lines, respectively (Figure 2.3).

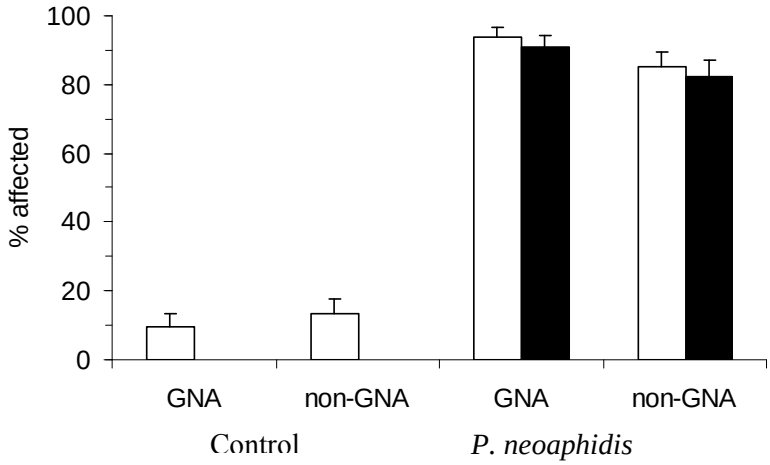

Figure 2.3: Mortality (□) and infection (■) for *Metopolophium dirhodum* on trangenic GNA or non-transformed wheat lines, either with or without inoculation by *Pandora neoaphidis* conidia. There was no infection in uninoculated groups of aphids. Means and SE pooled from three bioassays.

Plant lines and nymphal production

Mean nymphal production differed between bioassay runs (F = 3.33; df = 2, 267; P = 0.036). However, there was no difference in mean nymphal production by adult aphids either between GNA and parental lines (F = 0.00; df = 1, 267; P = 0.948) or between fungus-treated and control aphids (F = 1.11; df = 1 267; P = 0.293). Averaged over all bioassays and irrespective of whether aphids had been treated with *P. neoaphidis* conidia or not, mean nymphal production for *M. dirhodum* apterae was 18.6 nymphs aptera^{-1} (SE = 0.47) with the transgenic GNA line and 18.5 nymphs aptera^{-1} (SE = 0.47) with the parental line.

Plant lines and survival analysis

Data could not be pooled across bioassay runs as initial analyses showed differences between bioassays for aphids exposed to *P. neoaphidis* feeding on the transgenic GNA line (χ^2 = 17.76; df = 4; P < 0.01). In the first bioassay, infection occurred approximately one day earlier for aphids feeding on transgenic GNA, but no differences were found in subsequent bioassays and in general infection occurred at about five days after inoculation (data not shown).

Discussion

The study was carried out to investigate whether susceptibility of aphids to *P. neoaphidis* was affected if aphid hosts were feeding on a transgenic wheat line. Even though GNA may not cause direct toxicity, aphids under physiological stress caused by feeding on transgenic GNA may alter the performance of *P. neoaphidis*. Starvation stress in spotted alfalfa aphid, *Therioaphis maculata*, was shown to reduce infection by a related entomophthoralean fungus *Zoophthora radicans* (Milner and Soper, 1981). As far as we are aware, only one study has investigated transgenic plant – herbivore – entomopathogen tritrophic interactions. Johnson *et al.* (1997) observed that *Heliothis virescens* larvae adapted faster to Bt expressed in tobacco plants when exposed to conidia of the entomopathogenic fungus *Nomuraea rileyi*.

In previous studies with transgenic plants expressing GNA, the aphids *M. persicae* or *Sitobion avenae* and their arthropod natural enemies have shown no direct toxic effects on predators or parasitoids (Down *et al.* 2000; Couty *et al.* 2001a, b, c). However, detrimental changes were reported for two-spot ladybirds in life history parameters such as larval development and adult fecundity (Birch *et al.* 1999). In the current study, no adverse effects of GNA were observed against *P. neoaphidis*. This may have been a consequence of the aphid host, *M. dirhodum,* being unaffected by GNA, since there were no differences in either aphid mortality or fecundity between transgenic and untransformed wheat lines. This is in contrast with previous work with aphids such as *S. avenae* and *M. persicae* where several fitness parameters were significantly reduced (e.g. Gatehouse *et al.*, 1996). Alternatively, the absence of observed effects, either at the second (aphid) or third (fungus) trophic levels may have been due to the low levels of GNA in the wheat plants. Whilst most studies using artificial diet to deliver GNA have shown sublethal effects on aphids at concentrations of 0.01 – 0.15% w/v GNA (Sauvion *et al*. 1996), studies with transgenic plants have utilised plants with expression levels in the range of 1.0-1.5% total soluble protein (Gatehouse *et al*., 1996), although Stoger *et al*. (1999) demonstrated that wheat expressing lower levels of GNA (up to 0.2% protein) caused sublethal effects in *S. avenae*. However, transgene expression levels may not reflect amounts present in the phloem sap, which has been suggested for GNA in rice (Foissac *et al*., 2000). Hence. it is not possible to conclude whether the lack of GNA toxicity in the present study was a consequence of low levels of transgene expression, or insensitivity of either *M. dirhodum* or *P. neoaphidis* to GNA. Further studies are required to determine GNA levels in phloem and the potential susceptibility of a much wider range of aphid species than previously considered, which may have implications for developing integrated pest management strategies using this form of partial plant resistance.

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2.3 Insecticide resistance

Abstract

Bioassays were done to compare the susceptibility of different genotypes of the aphid *Myzus persicae* to *Pandora neoaphidis*. Aphid genotypes were chosen according to their insecticide resistance mechanism, either esterase - based or knock-down (kdr) resistance, and degree of resistance (high level of esterase, homozygous non-kdr, homozygous kdr). Two sets of experiments were done. In the first experiment, preliminary results indicated there were no differences in the virulence of two isolates to four insecticide-susceptible genotypes. In the second experiment using only one isolate, fungal virulence was greatest against the insecticide-susceptible genotype US1L, and least against *M. persicae* genotypes with high levels of esterase - based resistance.

Introduction

Insecticide resistance in the peach-potato aphid, *Myzus persicae*, has been studied extensively at Rothamsted Research. Various forms of resistance mechanisms have been identified but no information exists on how insecticide resistance may affect infection by fungal pathogens such as *Pandora neoaphidis*. Studies were carried out to determine virulence of three isolates against insecticide-susceptible *M. persicae* genotypes, followed by testing of one isolate against a range of susceptible, resistant and revertant genotypes.

Materials and Methods

Aphid cultures

Aphids of 10 genotypes (Table 2.3) were maintained separately on intact Chinese cabbage plants at 21°C with a 16:8 hour light:dark regime.

Fungal cultures

Two isolates were used in this study: NW 327, originally isolated from *Acyrthosiphon pisum* at Rothamsted, UK, in the 1970's, and NW 316, collected from infected *Microlophium carnosum* at Rothamsted in 1996. After retrieval from storage, the fungus was grown on Sabouraud dextrose agar supplemented with egg yolk and milk in Petri dishes (Wilding and Brobyn, 1980) and maintained in complete darkness at 20°C inside humid plastic boxes for three to four weeks before being used.

Table 2.3: *Myzus persicae* genotypes classified for resistance mechanism and degree of resistance

Clone	Resistant / susceptible	Resistance level	Resistance mechanism
US1L	susceptible	SS	Non Kdr non esterase
4255A	susceptible	SS	Non Kdr non esterase
4106A	susceptible	SS	Non Kdr non esterase
1076A	susceptible	SS	Non Kdr non esterase
800F	susceptible	SS R3	Non kdr esterase
1050A	susceptible	SS R3	Non kdr esterase
923A	susceptible	RR S revertant	Kdr non esterase
797J2 (rev)	susceptible	RR S revertant	Kdr non esterase
794J2	resistant	RR R3	Kdr esterase
2043B	resistant	RR R3	Kdr esterase

Bioassay procedure

Aphids were showered in groups of 10 on Chinese cabbage leaf discs mounted on 2% water agar. A 10 mm diameter round coverslip was placed next to aphids for dose estimation. Sporulating cadavers were inverted over the aphids of each genotype for different time periods (1, 2, 4 and 6 hours) to achieve a range of doses. Control aphids were held under the same conditions for the longest exposure time but received no conidia. The leaf discs were then removed from the agar and placed individually at the base of one week old Chinese cabbage plants and the aphids permitted to migrate onto the plants. Mortality was assessed daily for 7 days. Cover slips were fixed and stained using 10% cotton blue in lactophenol and mounted on microscope slides. Doses were estimated by counting the number of conidia in 20 fields of view per coverslip at 250X magnification.

In the first experiment the susceptibility of four insecticide-susceptible genotypes (US1L 4255A 4106A 1076A) to two isolates (NW316, NW327) of the fungus *Pandora neoaphidis* were compared on three occasions (both isolates were assayed simultaneously). In the second experiment, the susceptibility of eight genotypes (including two genotypes from the first experiment;) to isolate NW327 were compared on three occasions. These data allow for comparisons between insecticide-susceptible and resistant genotypes. Statistical analyses were carried out using the probit analysis facilities of GenStat (GenStat Committee, 2002).

Results

Data are still being analysed and only interim conclusions are presented. There appear to be no major differences in infection responses by the four insecticide-susceptible genotypes to *P. neoaphidis*, although there was a consistent trend for greater virulence of isolate NW316 compared with isolate NW327 (Figure 2.4). The results for the second experiment show some variability in response by genotypes, depending on resistance mechanism (Figure 2.5). The insecticide-susceptible genotype US1L was most susceptible to infection and genotypes with high levels of estrase based resistance were more tolerant of infection compared with US1L.

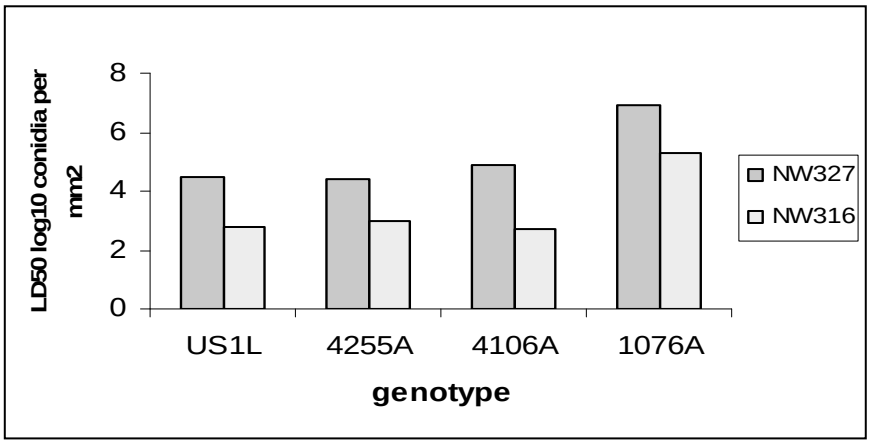

Figure 2.4: Variation in infection by susceptible *M. persicae* genotypes to *P. neoaphidis*

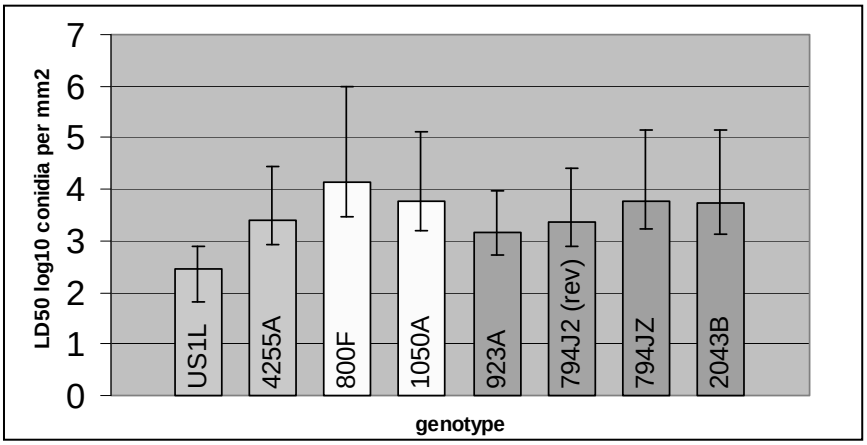

Figure 2.5: Variation in susceptibility of different *M. persicae* genotypes to isolate NW 327

Discussion

The results suggest there is variation in responses of insecticide-susceptible genotypes to infection by *P. neoaphidis*, but also that aphids with a high level of esterase resistance are also more tolerant to *P. neoaphidis*. Further studies are needed to determine if the outcome from this work, using the highest level of resistance, are also consistent with different resistance levels and genotypes of *M. persicae*. Attempts to

develop protocols for evaluating the role of behaviour in susceptibility of different genotypes and transmission in mixed populations were not successful due to the overall moderate - low susceptibility of *M. persicae*, with LD_{50} values of 200-1000 conidia per mm^2 for all genotypes.

Acknowledgements

The experimental work was done in collaboration with A. Sommer (Rothamsted Research).

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Objective 3 Develop discriminating "fingerprints" for field derived *P. neoaphidis* isolates

3.1 *Pandora neoaphidis* species-specific primers

Anna M. Tymon, Paresh A. Shah and Judith K. Pell (2004). PCR-based molecular discrimination of *Pandora neoaphidis* isolates from related entomopathogenic fungi and development of species specific diagnostic primers. Mycological Research, 108: IN PRESS.

Abstract

Studies were performed to assess the genetic variation amongst isolates of the aphid-pathogenic fungus *Pandora neoaphidis* (syn. *Erynia neoaphidis*). 37 isolates were examined, from a range of pest and non-pest aphid species, as well as 21 from eight other entomophthoralean species. Universal primers were used to amplify the ITS rDNA regions and all of the species tested produced discrete ITS groups, with the exception of *Conidiobolus* spp. Neighbour-joining analysis of the ITS2 regions from *P. neoaphidis*, *P. kondoiensis* and *Zoophthora radicans* demonstrated that these three species formed distinct groups with sequence identities of 58-82 % amongst the groups. An ITS size of 1100 bp was diagnostic for *P. neoaphidis*, while 1450 bp was characteristic of *P. kondoiensis*. ITS-RFLP analysis failed to yield intraspecific polymorphisms in any of the *P. neoaphidis* isolates screened, although it was useful in distinguishing between different entomophthoralean species. Some intraspecific variation in the ITS region was detected in some isolates of *Z. radicans* and *Conidiobolus* spp. We propose that two isolates previously identified as *P. neoaphidis* based on conidia morphology, are actually *P. kondoiensis* based on molecular studies. Sequencing analysis of the complete ITS region from *P. neoaphidis* and *P. kondoiensis* allowed species-specific primers to be developed for *P. neoaphidis* and *P. kondoiensis*. These were used to screen aphids infected in laboratory bioassays and from field collected samples, without prior isolation of the fungus. These primers are useful tools for quantifying the development of *P. neoaphidis* epizootics in aphid populations and for avoiding confusion with *P. kondoiensis*.

3.2 Molecular techniques to develop isolate-specific primers

Abstract

PCR-based DNA fingerprint analyses were undertaken to study intraspecific variation in *Pandora neoaphidis*. DNA was amplified from 30 isolates of *P. neoaphidis*, together with 6 species from closely related genera of Entomophthorales. The primers used included the following: ISSR (Inter Simple Sequence Repeat), ERIC (Enterobacterial Repetitive Intergenic Consensus) and RAPD (Random Amplification of Polymorphic DNA), and a random primer based on the universal M13 core sequence. Results indicated that *P. neoaphidis* isolates were highly polymorphic but they separated into a monophyletic group, compared with other Entomophthorales. UK isolates occupied two of three distinct clusters or sub-clades. One of these clusters was comprised of isolates obtained from the large nettle aphid, but no other correlations with aphid host were detected. ERIC, ISSR and RAPD analysis allowed the rapid genetic characterisation of different isolates and the generation of potential isolate and cluster-specific diagnostic DNA markers.

Introduction

The entomopathogenic fungus *Pandora neoaphidis* is quite variable in its morphological and physiological characteristics, but recent attempts to discriminate between isolates of this fungus using different sets of primers failed to demonstrate any intraspecific variation (Nielsen *et al*, 2001; Tymon *et al*, 2004). It is important to be able to distinguish between individual isolates, or genotypes, for monitoring and tracking isolates during field studies and to assess the amount of genetic variation among different isolates, which may have implications for the use of *P. neoaphidis* for biological control.

The aims of this study were to determine whether ERIC, ISSR and RAPD primers were effective in detecting genetic variation among *P. neoaphidis* isolates, and to determine whether specific genotypes could be correlated with aphid host and/ or geographical origin. The development of isolate-specific DNA markers for field studies was also attempted.

Materials and Methods

Isolates

Thirty *P. neoaphidis* isolates with a worldwide distribution infecting both pest and non-pest aphid species were tested (Table 3.1). Isolates from six other closely related entomophthoralean species were also included for comparison (Table 3.2).

DNA techniques

Fungal cultivation and DNA extractions: Details are given in Tymon *et al.* (2004).

Oligonucleotide primers: Details of the four ISSR primers, two ERIC primers, four RAPD primers and the universal M13 core sequence primer are given in Table 3.1.

PCR: DNA amplifications were carried out in a Hybaid Express thermocycler (Hybaid, UK). Each PCR reaction was performed at least twice in 20 µl volumes containing 1 µl of diluted DNA. Cycling conditions were optimised and 1 Kb and 100 bp size markers were included in gels. Gels were stained with ethidium bromide (0.1 µg µl $^{-1}$) and photographed using a digital camera. Bands were only scored if they were reproducible and appeared in at least two separate PCR reactions. No weight was given to stronger bands. Bands were scored for presence (1) or absence (0) in each isolate for each primer combination.

Data analysis: The freeware program FreeTree (Hampl *et al.*, 2001) was used to generate phylograms using both neighbour-joining analysis of similarity matrices created using Jaccard's coefficient and also UPGMA (unweighted pair group method with arithmetic average) analysis. One hundred bootstrapped replicates, also conducted in FreeTree, were carried out on the original datasets to test the tree topologies obtained. Trees based on separate ISSR, ERIC or RAPD data were produced and rooted using *C. obscurus* as the outgroup. Finally, the data were pooled from all eleven primers to produce a consensus tree, again using *C. obscurus* as the outgroup. Tree files were read using TREEVIEW (Page, 1996).

Cloning and sequencing of PCR amplified polymorphic markers: Polymorphic bands which were unique for an isolate or cluster were excised from agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen Ltd, UK) used according to manufacturers recommendations. If DNA concentrations were low, bands were re-amplified from the purified fragments, gel-purified for a second time then ligated into T-tailed pBluescript SK. Following transformation of DH5α bacterial cells, plasmid preparations were produced from selected clones using the Wizard® PlusSV Minipreps DNA Purification System (Promega, UK). Where plasmids were obtained, sequencing of DNA inserts was carried out using the ABI Prism BigDye Cycle Sequencing Kit (PE Applied Biosystems Division, Warrington). Inserts were sequenced in both directions using universal forward and reverse primers. Based on the sequences obtained, 14 primers combinations were tested against a range of *P. neoaphidis* isolates to assess their specificity.

Results

All the primers produced reproducible bands but the number of bands which were amplified varied greatly (Table 3.3). The greatest number of bands was generated using ERIC primers from all five primers used (Figures 3.1-3.3), with a total of 54 reproducible bands scored. Eighteen of the 54 bands (33.3 %) were polymorphic among the different isolates tested. Some of these bands were shared by groups of isolates and others appeared to be isolate specific. The M13 random primer produced the least discernable and smallest number of scorable bands. However 12 of the 18 bands (66.7%) were polymorphic. Two polymorphic bands appeared specific for a group of four UK isolates NW 356, NW 427, NW 416 and NW 422 (Figure 3.3 A).

Of the four ISSR primers tested, CCA produced 36 scorable bands, of which 32 were polymorphic (88.9%). Only isolate NW 314 failed to amplify any products with this primer (Figure 3.2 A). Smaller numbers of bands were generated with the GACA, ACA, and CGA primers (Table 3.3).

The numbers of bands produced by the four RAPD primers (OPA-1, OPA-11; OPG-11 and OPG-13) varied slightly, but were similar to those produced by the ISSR primers (Table 3.3). An example of OPG-13

amplification is shown (Figure 3.2 B). The banding patterns of eight UK plus four worldwide isolates, amplified by primer OPA-11, show these isolates could be easily distinguished from one other by the presence or absence of specific diagnostic DNA bands (Figure 3.3 B).

Table 3.1: List of *Pandora neoaphidis* isolates used in the study

Isolate code no.[1]	Aphid host	Location	Year[2]
Isolates from legume crop aphids			
ARSEF 1985	*Aphis fabae*	France	1985
NW 283	*A. fabae*	Rothamsted, UK	1992
ARSEF 1603	*Acyrthosiphon pisum*	Spain	1984
ARSEF 1609	*A. pisum*	France	1984
ARSEF 2872	*A. pisum*	USA	1989
NW 327	*A. pisum*	Rothamsted, UK	1997
NW 434	*A. pisum*	Rothamsted, UK	2001
Isolates from brassica crop aphids			
ARSEF 1600	*Myzus persicae*	Portugal	1984
ARSEF 3251	*M. persicae*	USA	1990
NW 343	*M. persicae*	Rothamsted, UK	1998
ARSEF 2123	*Brevicoryne brassicae*	Bosnia & Herzegovina	1985
NW 314	aphid on brassica leaf	Rothamsted, UK	1995
Isolates from cereal crop aphids			
ARSEF 3081	*Diuraphis noxia*	USA	1990
ARSEF 3126	*D. noxia*	France	1990
ARSEF 3174	*D. noxia*	France	1990
ARSEF 2875	*Metopolophium dirhodum*	USA	1989
NW 195	*M. dirhodum*	Belgium	1985
NW 284	*M. dirhodum*	Rothamsted, UK	1992
NW 286	*M. dirhodum*	Rothamsted, UK	1992
ARSEF 5403	*Sitobion avenae*	Denmark	1995
KVL 630	*S. avenae*	Denmark	1995
ARSEF 1604	*Sitobion sp.*	France	1984
Isolates from non-pest aphids			
ARSEF 833	*Hyperomyzus lactucae*	Chile	1981
ARSEF 835	*H. lactucae*	Australia	1982
NW 316	*Microlophium carnosum*	Rothamsted, UK	1996
NW 356	*M. carnosum*	Hatfield, UK	1999
NW 416	*M. carnosum*	Rothamsted, UK	2000
NW 422	*M. carnosum*	Rothamsted, UK	2000
NW 427	*M. carnosum*	Sharnbrook, UK	2000
NW 415	*Microlophium primulae*	St. Albans, UK	2000

[1] NW indicates isolates held at Rothamsted, UK; ARSEF isolates from R. Humber, USDA-ARSEF, USA; KVL isolate from J.Eilenberg, KVL, Denmark.

[2] Year of collection and liquid nitrogen preservation of isolate.

Table 3.2: List of related entomophthoralean fungi used in study

Isolate code no.	Fungal isolate	Location	Year[1]	Insect order
ARSEF 3139	*Conidiobolus obscurus*	USA	1990	Aphididae
NW 344	*Pandora blunckii*	Mexico	1998	Plutellidae
ARSEF 134	*Pandora delphacis* (ex-neotype)	Japan	1975	Delphacidae
ARSEF 828	*Pandora kondoiensis (*ex-isotype)	Australia	1980	Aphididae
ARSEF 5707	*P. kondoiensis*	South Africa	1998	Aphididae
ARSEF 5708	*P. kondoiensis*	South Africa	1998	Aphididae
ARSEF 199	*Pandora nouryi*	USA	1977	Aphididae
NW 378	*Zoophthora radicans*	Mexico	1999	Plutellidae

[1] Year of collection and liquid nitrogen preservation of isolate.

Table 3.3: DNA fragments amplified with ISSR, ERIC, M13 and RAPD primers

Primer	Primer sequence[§]	Tm (°C)	Annealing temperature (°C)	Total number of fragments[¶]	Polymorphic fragments[*]	Size range (bp)
ISSR - CCA	5'- DD (CCA)5	56.8	51	36	32 (88.9 %)	250 – 2500
ISSR - ACA	5'- BDB (ACA)5	48.3	51	31	23 (74.1 %)	250 – 1700
ISSR - CGA	5'- DHB (CGA)5	59	48	30	13 (43.3 %)	300 – 3500
ISSR - GACA	5'- (GACA)4	49.2	45	24	21 (87.5 %)	400 – 1900
RI CIRE[@]	5'- CACTTAGGGGTCCTCGAATGT	59.8	45.5	54	18 (33.3 %)	<100 – 2000
ERIC R2[@]	5'- AAGTAAGTGACTGGGGTGAGCG	62.1	45.5	-	-	-
M13	5'- TTATGTAAAACGACGGCCAGT	55.9	45	18	12 (66.7 %)	300 –14 00
OPA-1	5'- CAGGCCCTTC	34	35	30	19 (63.3 %)	200 – 1250
OPA-11	5'- CAATCGCCGT	32	35	32	25 (78.1 %)	300 – >3000
OPG-11	5'- TGCCCGTCGT	34	35	24	11 (45.8 %)	400 –1900
OPG-13	5'- CTCTCCGCCA	34	35	30	20 (66.7 %)	240 – 1700

[§] Designations for degenerate sites are: H (A, T or C); B (G, T or C); Y (G, A or C) and D (G, A or T).

[@] RI CIRE and ERIC R2 used as a primer pair.

[¶] Total numbers of reproducible bands scored.

[*] Percentages of polymorphic fragments are given in parentheses

Figure 3.1: ERIC-PCR fingerprints of *P. neoaphidis* isolates and other related entomopathogenic fungi. Isolates were: Lanes 1-12 *P. neoaphidis* (Pn) UK isolates NW 284, 286, 314, 343, 415, 316, 327, 434, 416, 422, 427, and 356. Lanes 13 and 35 = *C. obscurus* (Co), lanes 14-16 = *P. kondoiensis* (Pk) isolates ARSEF 5708, 5707 and 828. *P. delphacis* (Pd) = lane 17; *Z. radicans* (Zr) = lane 18. Lanes 19-34 *P. neoaphidis* (Pn) isolates NW 195, KVL 630, ARSEF 835, 1609, 3174, 833, 2872, 3251, 2875, 3081, 5403, 2123, 1603, 1600, 1985 and 3126. *P. nouryi* (Pno) = Lane 36. M is a 1 Kb size marker; M^1 is a 100 bp marker. Arrows within gel lanes indicate a range of polymorphic bands between *P. neoaphidis* isolates.

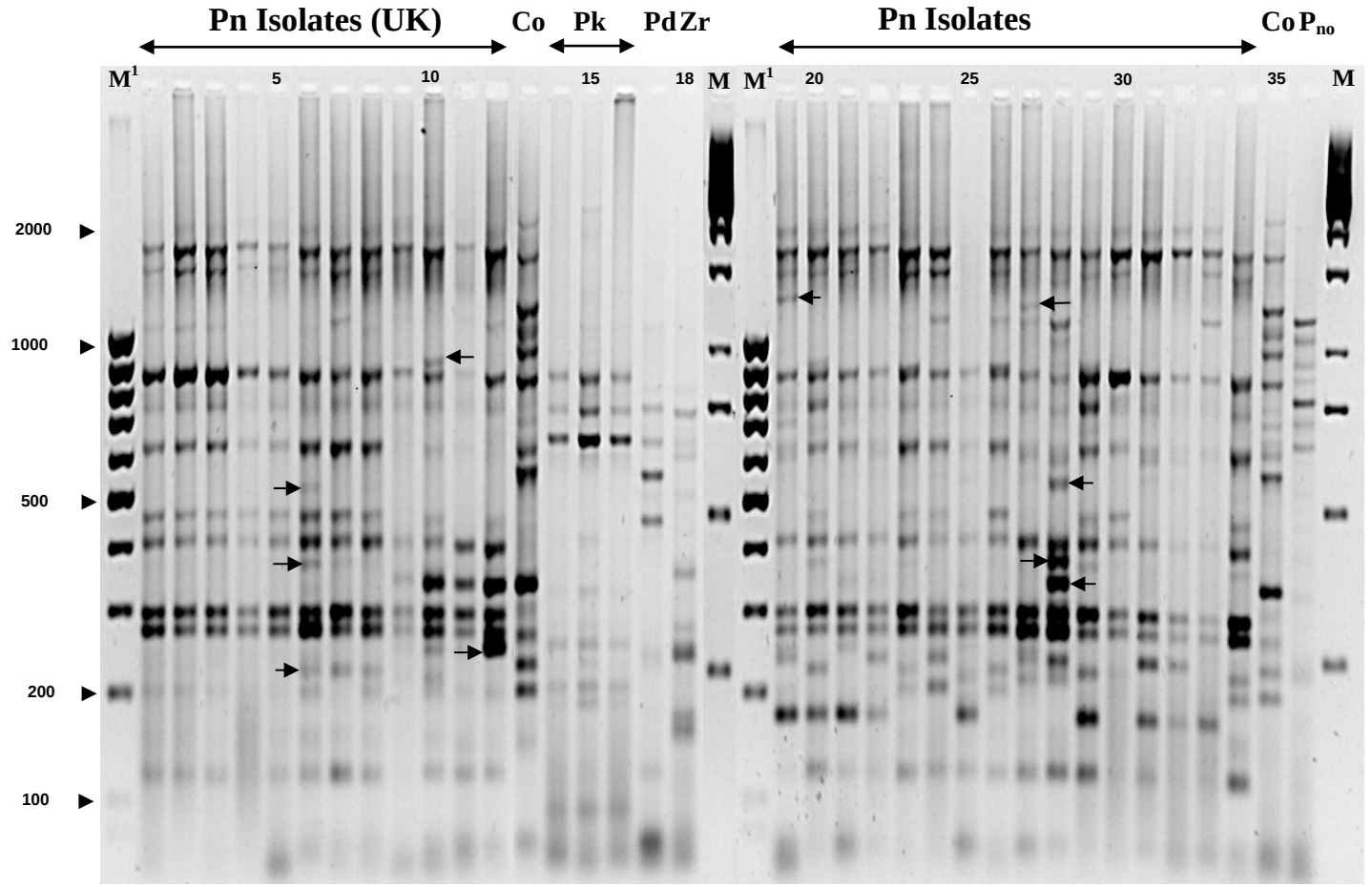


Figure 3.2: Amplification of isolates using: (A) CCA-ISSR primer and (B) OPG-13 RAPD primer. Lanes 1-30 *P. neoaphidis* (Pn) isolates; 1-13 = UK isolates NW 283, 282, 286, 314, 343, 415, 316, 327, 434, 416, 422, 427, 356. Lanes 14-30 = worldwide isolates NW 195; KVL 630; ARSEF 835, 1609, 3174, 833, 2872, 3251, 2875, 3081, 5403, 2123, 1603, 1600, 1985, 1604 and 3126. Lane 31 = *C. obscurus* (Co); lanes 32-34 = *P. kondoiensis* (Pk) isolates ARSEF 5708, 5707 and 828; lane 35= *P. delphacis* (Pd); lane 36= *P. nouryi* (Pno); lane 37= *Z. radicans* (Zr) and lane 38= *P. blunckii* (Pb). M= 1 Kb size marker. Arrows within gel lanes indicate polymorphic bands.

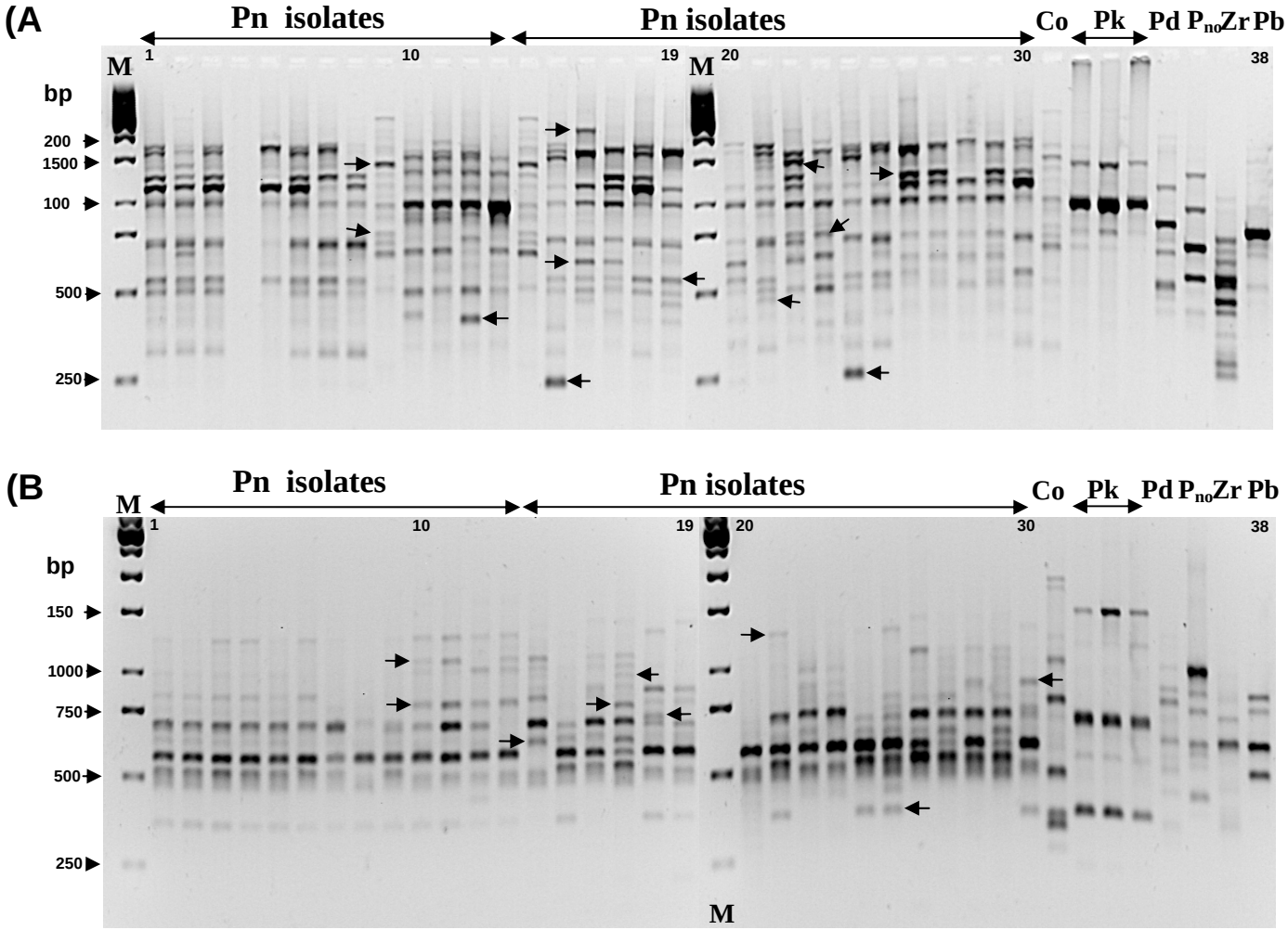
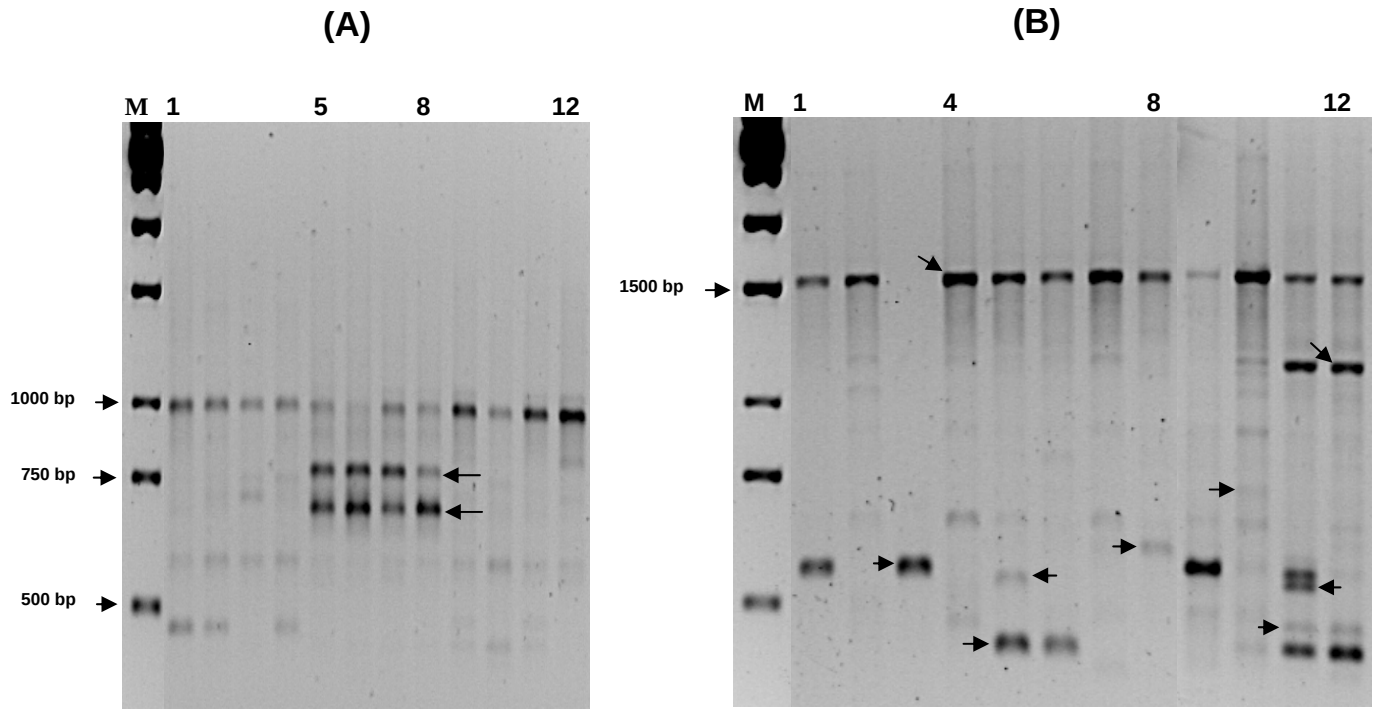


Figure 3.3: Intraspecific variation detected using: (A) M13 random primer (B) OPA-11 RAPD primer. (A): Lanes 1-12 *P. neoaphidis* isolates; 1-8 = UK isolates NW 415, 316, 327, 434, 416, 422, 427 and 356. Lanes 9-12 = worldwide isolates NW 195; KVL 630; ARSEF 835 and 1609. M= 1 Kb size marker. (B): 1-8 = UK isolates (as in A); 9-12 = ARSEF 2872, 3251, 2875 and 3081. Arrows indicate polymorphic bands.



Phylogenetic analysis

A consensus tree produced from Neighbour Joining analysis of the pooled PCR fingerprint data demonstrated that *P. neoaphidis* isolates were clearly distinguished from other related entomophthoralean species, producing a distinct monophyletic group, supported by bootstrap percentages of 100 % (Figure 3.4). This monophyletic group further separated into three distinct sub-clades as follows: Sub-clade 1 contained the UK isolates NW 356, NW 427, NW 416 and NW 422. Sub-clade 2 consisted of the remaining nine UK isolates plus ARSEF 3126 in group a, and KVL 630, ARSEF 3251, ARSEF 2123, ARSEF 5403, ARSEF 3174 and ARSEF 833 in group b. Sub-clade 3 comprised isolates ARSEF 1985, ARSEF 1603, ARSEF 1600, ARSEF 1604, ARSEF 2875 and ARSEF 3081 in group a, and ARSEF 2872, ARSEF 835, ARSEF 1609 and NW 195 in group b. The three *P. kondoiensis* isolates used for comparison also separated into a distinct clade supported by 100 % bootstrap values.

The consensus tree (Figure 3.4) was similar to individual trees produced using either ERIC, combined RAPD or combined ISSR results (results not shown). Although there were some slight changes in isolate order found in sub-clades 1 and 3 when individual primer results were analysed, the four isolates in 1 always grouped together, with the UK isolates always separating only into sub-clades 1 and 3.

UPGMA analysis on the combined PCR data set, using the FreeTree software program, provided a very similar consensus tree to that obtained using Neighbour-Joining analysis (results not shown), with *P. neoaphidis* separating into a monophyletic group which further split into three distinct clusters. Only the order of some isolates within sub-clades 2 and 3 varied. The *P. kondoiensis* isolates also produced a separate clade supported by 100% bootstrap values in this analysis.

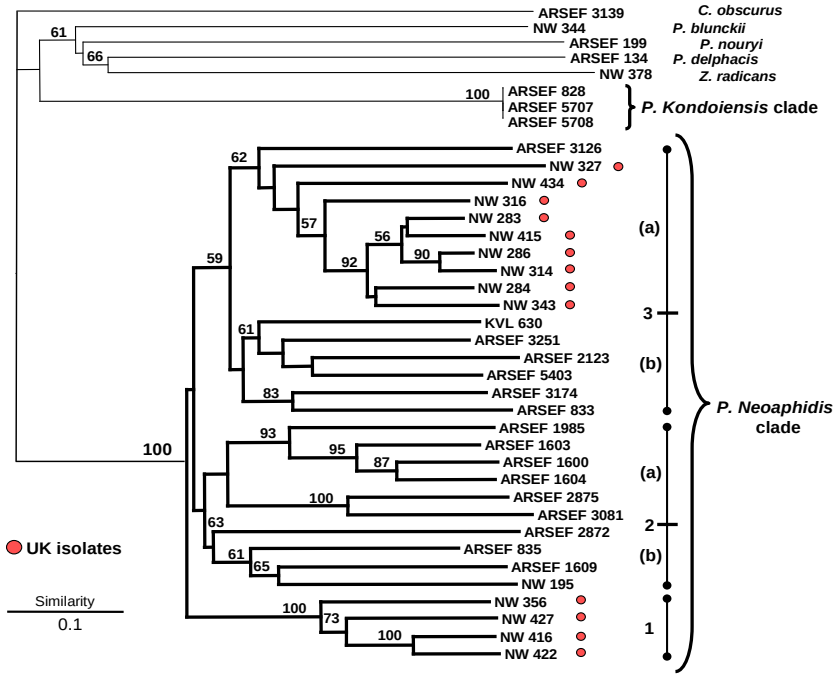


Figure 3.4: Phylogenetic relationships inferred from Neighbour-Joining analysis from a similarity matrix created using Jaccard's co-efficient, of the presence or absence of PCR products. The branches connecting the *P. neoaphidis* isolates are shown with bolder lines. Only bootstrap values above 55% are shown. Sub-clades are indicated by numbers in bold.

Analysis of cloned polymorphic DNA fragments

Several attempts were made to clone a variety of polymorphic DNA bands (unique to isolates/ clusters), amplified using a range of primers. It was necessary to re-amplify many of the purified PCR bands in order to produce DNA of sufficient quantity/quality for cloning purposes. The *P. neoaphidis* isolates chosen for screening were from each of the identified group clusters (Figure 3.4). Using RAPD primer OPA-11, fragments amplified from isolates NW 356, ARSEF 833 and ARSEF 2875 were successfully cloned as was an OPG-13 fragment from isolate ARSEF 1609. It was found that the sequences of the OPA-11 fragments from NW 356 and ARSEF 833 were very similar, only differing at their 5'-ends (data not shown).

Several attempts were made to clone and sequence three polymorphic fragments produced by the random primer M13 from isolates NW 356, NW 416 and NW 422. Primers based on these sequences were not produced and tested during the project because the fragments were difficult to clone and sequences.

Two ERIC bands from NW 316, one from NW 327 and one from NW 356 were re-amplified, cloned and sequenced. It was found that the sequences from the two NW 316 bands were identical, indicating that a mixed DNA fragment was probably used in the cloning attempts. All attempts to clone purified fragments generated by the ISSR primer GACA failed. It also proved difficult to purify any of the other ISSR polymorphic fragments due to mixed fragments being reamplified each time (data not shown). However, only one pair of primers showed potential to discriminate between isolates. These primers (and additional primers) require further developmental work and optimisation before they can be used to identify particular isolates in infected aphids.

Discussion

This study has shown for the first time that both ERIC and ISSR fingerprinting are powerful tools for differentiating among isolates of *P. neoaphidis*. The range of different amplification products obtained with all eleven of the primers clearly demonstrated high levels of intraspecific variation among *P. neoaphidis* isolates. The banding patterns produced by *P. neoaphidis* isolates were distinctly different to those produced by other entomophthoralean species, with the numbers of products being generally much greater.

Overall we confirm that *P. neoaphidis* is a monophyletic group. Previous phylogenetic analyses based on small subunit ribosomal DNA sequences demonstrated that there were two clades within the family Entomophthoracea, containing either the genus *Pandora* or *Zoophthora* (Jensen *et al.*, 1998). Our phylogenetic analysis demonstrates that *P. neoaphidis* can clearly be distinguished from other related entomophthoralean species, producing a distinct monophyletic group which was sub-divided into three distinct clusters or sub-clades. In addition we were able to separate *P. kondoiensis* into a separate clade, which supports previous work on these groupings (Tymon *et al.*, 2004).

A discrete group of four UK *P. neoaphidis* isolates obtained from nettle aphid *M. carnosum* were detected using primer M13, with two very distinct polymorphic bands being generated. This separate grouping was also demonstrated in the phylograms produced using the individual and combined datasets, with only these four UK isolates separating into sub-clade 1. The only other *M. carnosum* isolate tested, NW

316, was found to separate into sub-clade 3. Interestingly, although NW 316 was isolated from *M. carnosum* it was passaged through the pea aphid, *A. pisum*, which may have led to some genetic alteration in this isolate. However, apart from sub-clade 1, no relationships were found between aphid host species and isolate groupings.

Our data did not demonstrate the broad geographical separations into North American and European *P. neoaphidis* isolates reported by Nielsen *et al.* (2001). Possible reasons include the greater number and types of primers used in our study and our inclusion of isolates from a broader range of countries such as Australia and Chile and the number of North American isolates screened.

The lack of a relationship between host species and geographical origin would suggest that *P. neoaphidis* may be ecologically mobile and isolates are likely to cross-infect several aphid host species. Our molecular data supports evidence from laboratory bioassays which have demonstrated that individual *P. neoaphidis* isolates can infect more than one aphid species and isolates from non-pest species were often highly virulent to pest aphid species (e.g. Sierotzki *et al.*, 2000; Shah *et al.*, 2004).

With such a high degree of polymorphism detected within *P. neoaphidis*, certain DNA fragments were unique for particular isolates. For example the two polymorphic fragments amplified by primer M13 were diagnostic for isolates in sub-clade 1. Also, certain combinations of banding patterns would allow specific isolates from pest and non-pest hosts to be tested together, to see if one or other would preferentially infect selected aphid species using just two diagnostic primers i.e. M13 and OPA-11.

With the production of species-specific markers for *P. neoaphidis*, it should be possible to design diagnostic primers based on sequence information from these polymorphic fragments. This was attempted during the project but was unsuccessful. Construction of isolate-specific diagnostic primers would greatly enhance our ability to understand fundamental questions on possible relationships between genetic variation and biological traits (e.g. virulence), spatial structure, ecological niche separation, the distribution of genotypes amongst different aphid hosts, and persistence of isolates with different attributes in agro-ecosystems. On a practical level, isolate-specific fingerprints would permit studies on tracking the dispersal of isolates during epizootics produced naturally or by augmentation.

3.3 Use if molecular fingerprints

It was not possible to develop molecular probes in the duration of the project. Further developmental work is necessary to achieve this goal. Samples of infected aphids were collected in 2002 and are currently stored in ethanol at 4°C for evaluation should isolate-specific molecular probes for particular *P. neoaphidis* isolates become available. Virtually no infection by *P. neoaphidis* was found in aphids during 2003 (the year in which it was planned to use the probes) because of the exceptionally hot conditions during the spring and summer months.

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3.4 Effects of fungicides on *P. neoaphidis*

Abstract

The effects of ten different fungicides were evaluated *in vitro* on a number of different isolates of *Pandora neoaphidis*. The fungicides chlorothalonil, dichlofluanid, fenhexamid, prochloraz and carbendazim caused complete inhibition of germination of conidia at recommended rates. At lower concentrations, adverse effects on germination were much greater with protectant fungicides than with the systemic ones. This study demonstrated that isolates vary in their sensitivity, and that possible adverse effects are complex, depending on the chemical and *P. neoaphidis* stage under consideration.

Introduction

Studies are necessary to determine the degree of compatibility between fungicides and fungal entomopathogens, if integrated pest and disease control is to be achieved in agroecosystems. Previous laboratory and field studies indicated that certain fungicides, such as benomyl, are detrimental to mycelial growth of various Entomophthorales, while other compounds, such as chlorothalonil, may have little or no effect (Hall and Dunn, 1959; Soper *et al.*, 1974; Lagnaoui and Radcliffe, 1998). Wilding and Brobyn (1980) found that systemic and non-systemic fungicides inhibited germination of *Pandora neoaphidis* conidia, and some compounds (e.g. benomyl, maneb) also inhibited infectivity and/ or discharge of conidia from infected aphid cadavers.

The aims of the current study were to determine toxicity of a range of protectant and systemic fungicides on mycelial and conidia stages of *P. neoaphidis*.

Materials and Methods

Fungal cultures and fungicide compounds

Six isolates were used for studies (Table 3.4). After retrieval from liquid nitrogen, isolates were cultured on Sabouraud's dextrose agar supplemented with egg yolk and milk (SEMA) at 20 °C for three to four weeks before being used. Ten compounds were obtained from suppliers and covered a range of "old" and "new" products (Table 3.5).

Screening fungicides for mycelial inhibition

Radial growth of isolate NW 354 was assessed at concentrations of 1 and 10 ppm of each fungicide incorporated into SEMA plates. Three plugs (3 mm diameter) were placed in each Petri dish, and there were three replicate dishes for each concentration of each fungicide. After 14 days incubation, two diameters, at right angles, were measured in each Petri dish. The means were averaged for replicates and inhibition was expressed as a percentage of growth on control dishes, which received distilled water instead of fungicide.

Table 3.4: List of *Pandora neoaphidis* isolates used in fungicide testing experiments

Isolate	Host aphid
NW 286	*Metopolophium dirhodum*
NW 314	aphid on brassica leaf
NW 354	*Amphorophora rubi*
NW 356	*Microlophium carnosum*
KVL 630	*Sitobion avenae*

Table 3.5: List of fungicide compounds used in testing against *P. neoaphidis* isolates.

Compound	Chemical family	Field dose (ppm)	Manufacturer
Protectant			
Chlorothalonil	Aromatic hydrocarbon	2200	BASF AG
Maneb	Dithiocarbamate	6800	Rohm & Has
Dichlofluanid	Phthalimide	1100	Bayer
Fenhexamid	Hydroxyanilide	3750	Bayer
Systemic			
Carbendazim	Benzimidazole	2500	BASF AG
Prochloraz	Imidazole	2000	AgroEvo
Metalaxyl*	Phenylamide	750	Novartis
Azoxystrobin	Strobilurin	1250	Zeneca
Epoxiconazole*	Triazole	310	BASF AG
Fenpropimorph	Morpholine	1900	BASF AG

* experimental compounds from manufacturers

Effects of fungicides on conidia germination

Five fungicides (dichlofluanid, chlorothalonil, fenhexamid, prochloraz, carbendazim) were used at field rates incorporated into SEMA medium. Plugs from Petri dish cultures of isolate NW 356 were

suspended from the lid of each dish so that conidia were discharged onto SEMA for one hour. Conidia were then incubated for one, three and 24 hours. There were three replicate dishes for each fungicide and each time period. At these times, ca. 100 conidia were examined for germination after staining with lactophenol cotton blue under a light microscope. Conidia were assessed for germ tubes and / or empty "ghosts". Germination was expressed as a percentage of germination in control dishes, which were treated with distilled water.

Differences between isolates to azoxystrobin

Radial growth of isolates NW 286, NW 314, NW 356 and KVL 630 was assessed on SEMA medium incorporating azoxystrobin at 0.1, 1 or 10 ppm, with distilled water as a control. One plug was placed in the centre of each Petri dish and five replicate dishes were used for each concentration and isolate. After 14 days incubation, radial growths were measured and expressed as percentage inhibition of controls. The 100 ppm concentration was 3- to 68-times lower than recommended field rates of the various compounds (Table 3.5).

Results

Screening fungicides for mycelial inhibition

Azoxystrobin, carbendazin and prochloraz completely inhibited mycelial growth at 100 ppm (Figure 3.5), while there was 20-40% inhibition with other fungicides at this concentration. Stimulation of mycelial growth was observed at 1 ppm for carbendazim, chlorothalonil, dichlofluanid and fenhexamid, and for metalaxyl at both 1 and 100 ppm.

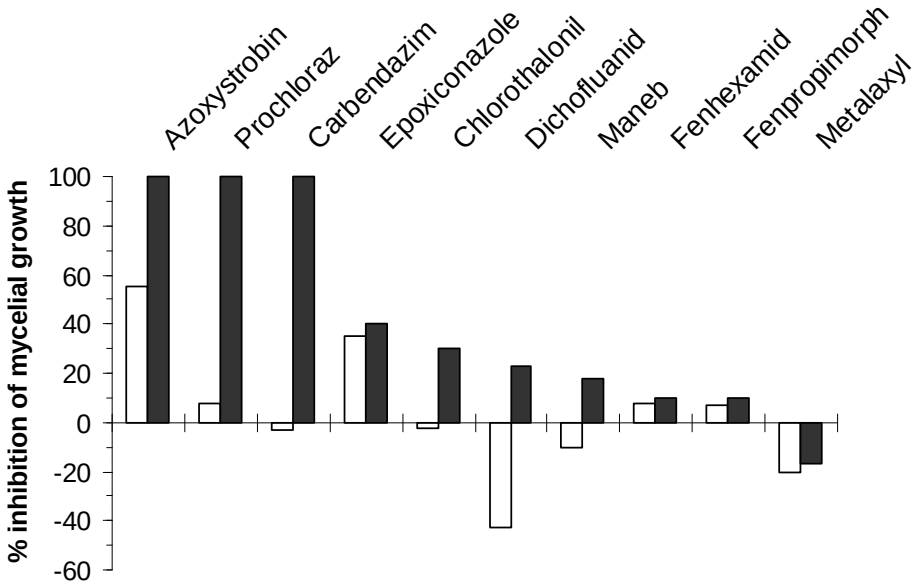

Figure 3.5: Effect of ten fungicides at 1 ppm (□) and 100 ppm (■) active ingredient on mycelial growth of isolate NW 354. Negative values indicate enhancement of growth.

Effects of fungicides on conidia germination

All fungicides tested at field rates completely inhibited germination of NW 356 at all time points used in evaluations (results not shown).

Differences between isolates to azoxystrobin

There was evidence for a dose-response effect in mycelial inhibition by different isolates to this fungicide (Figure 3.6). Of the four isolates tested, NW 356 was least affected at 0.1 and 1 ppm azoxystrobin and exhibited some growth at 10 ppm.

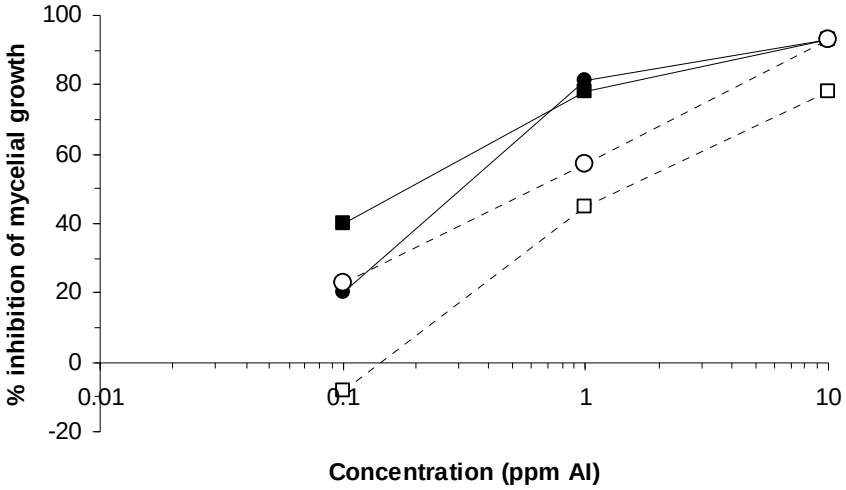

Figure 3.6: Effect of azoxystrobin on mycelial growth of *P. neoaphidis* isolates NW 286 (■), NW 314 (●), NW 356 (□) and KVL 630 (○)

Discussion

From Petri dish tests of mycelial growth, azoxystrobin was found to be the most toxic of the compounds tested, followed by prochloraz, carbendazim and epioxiconazole. Stimulatory effects at low concentrations (much less than field rate) were found with dichlofluanid, maneb and metalaxyl. Carbendazim, dichlofluanid and maneb inhibited mycelial growth of the ascomycetes *Beauveria bassiana*, *B. brongniartii* and *Lecanicillum lecanii* (Vyas *et al.*, 1990; Hassan *et al.*, 1991). Chlorothalonil was not considered to be harmful to *P. neoaphidis* even at twice its field rate in laboratory tests (Lagnaoui and Radcliffe, 1998) but was found to be moderately inhibitory in the current study which may be due to differences in the isolates used. In agreement with our results, metalaxyl was found to stimulate growth of *Metarhizium*, *Beauveria* and *Lecanicillium* spp. (Majchrowicz and Poprawski, 1993).

Results from experiments with azoxystrobin indicated that *P. neoaphidis* isolates varied in their sensitivity. Interestingly, NW 356, originally obtained from nettle aphid, was more tolerant of this fungicide than the other three isolates obtained from pest aphids. Allied with similar conclusions from virulence, temperature and molecular studies, it is evident that intra-specific variation occurs with *P. neoaphidis* but factors driving differentiation are unknown at present. No sexual (meiotic) stages have been recorded for this entomopathogen, which indicates that variation probably arises from mitotic mutations and mechanisms

such as heterothallism, where exchange of nuclear material occurs between different mating types, although evidence for mating types is also lacking.

In practical terms, most fungicides are likely to have a harmful effect on *P. neoaphidis*. Since field margins should not receive any pesticide inputs, the loss of fungal inoculum in crop areas sprayed with fungicides should be supplemented by dispersal of *P. neoaphidis* from field margin and other non-crop areas.

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3.5 Growth and enzyme characteristics of *Pandora neoaphidis*

Abstract

Investigations into the effects of varying temperature and media on the growth rates of seventeen isolates of the fungal entomopathogen *Pandora neoaphidis* and one isolate of *P. kondoiensis* were done. Isolates did not grow at temperatures of 30°C and there was limited growth at 4°C. The optimal temperatures for growth for the majority of isolates was between 18 and 22°C. Some isolates also grew reasonably well at 10-15°C. *Pandora kondoiensis* did not grow at 25°C in contrast to several *P. neoaphidis* isolates. Growth on a semi-defined medium at 20°C was also examined where *P. kondoiensis* had the strongest growth on semi-defined

media. Semi-quantitative enzyme analysis revealed some differences between intra- and extra-cellular enzymes in seven *P. neoaphidis* and one *P. kondoiensis* isolate.

Introduction

The entomopathogenic fungus *Pandora neoaphidis* is one of the most common fungal pathogens of aphids in temperate regions; the pathogen also causes natural epizootics in aphid populations and has potential application as a biocontrol agent (Pell *et al.*. 2001). In this study we report on the effects of temperature on growth of *P. neoaphidis* and *P. kondoiensis.* We also examined the effects of a semi-defined medium on growth characteristics. The aims were to identify isolates with varying temperature optima (especially growth at low temperatures), and to determine any differences in intra- and extra-cellular enzyme profiles of selected isolates, which may indicate differences in biological function.

Materials and methods

Fungal cultures

A total of 17 *P. neoaphidis* isolates and one isolate of *P. kondoiensis* were obtained from a range of pest and non-pest aphid species (Table 3.7). To produce inocula for experiments, isolates were grown on Sabouraud dextrose agar supplemented with milk and egg yolk (SEMA: Wilding and Brobyn, 1980), at 20°C in darkness.

Table 3.7: List of isolates used in studies on biological characteristics.

			Isolates used in studies:		
Isolate	Host	Country	SEMA growth rates	SDEM growth rates	Api-Zym analysis
NW 327	*Acyrthosiphon pisum*	UK	+	+	+
NW 428	*A. pisum*	UK			
NW 438	*A. pisum*	UK		+	
NW 283	*Aphis fabae*	UK			
ARSEF 5374	*Brevicoryne brassicae*	Denmark	+		
NW 314	aphid on brassica leaf	UK	+		+
ARSEF 3251	*Myzus persicae*	USA	+		
NW 343	*M. persicae*	UK	+		
NW 195	*Metopolophium dirhodum*	Belgium	+		
NW 284	*M. dirhodum*	UK			
ARSEF 5708*	*M. dirhodum*	South Africa	+	+	+
KVL 630	*Sitobion avenae*	Denmark	+		+
NW 316	*Microlophium carnosum*	UK	+		
NW 356	*M. carnosum*	UK	+	+	+
NW 416	*M. carnosum*	UK	+	+	
NW 415	*M. primulae*	UK	+		
NW 354	*Amphorophora rubi*	UK		+	+
ARSEF 833	*Hyperomyzus lactucae*	Chile	+		+

All isolates of *P. neoaphidis* except * *P. kondoiensis*

Effects of temperature on growth on SEMA

Growth characteristics on SEMA were examined for 13 *P. neoaphidis* and one *P. kondoiensis* isolate (Table 3.7). Using a 6mm-diameter, flame-sterilised, cork borer, single plugs were excised from the edges of established cultures, and placed individually (colony side up) at the centre of 9 cm, triple vented Petri dishes. Three replicate plates per isolate were used at each of eight constant temperatures (4, 10, 15, 18, 20, 22, 25 and 30° C) and grown in darkness. The growth of each isolate over time was estimated in at least two separate experiments.

Vegetative radial growth was measured every 2-3 days for 35-40 days. Colony diameters were marked and measured using a plastic ruler. The mean of the four lines was used to obtain the radial growth at every time point and temperature for each replicate. The mean growth (mm) from each set of replicate plates was then averaged to obtain the accumulated growth over time for each isolate. Accumulated radial growth (mm) was plotted against incubation period to calculate the growth rate at each temperature. Different combinations of isolates were assessed using an alpha design (incomplete blocking) on five separate occasions.

Growth on semi-defined medium

A semi-defined medium (SDEM; Gray *et al*. 1990) was used to examine the growth rate of five isolates of *P. neoaphidis* and one isolate of *P. kondoiensis* at 20 °C (Table 3.7). Cultures were placed inside sealed plastic boxes as before and radial growth was measured over a period of 36 days. Accumulated radial growth was measured and estimated as above.

Enzyme analysis

A semi-quantitative method using an Api-Zym kit (BioMérieux, France) was used to investigate the activity of nineteen enzymes in seven *P. neoaphidis* isolates and one *P. kondoiensis* isolate (Table 3.7). Isolates were grown in liquid culture (see Section 1.3 this report). To measure extra cellular enzymes released during the incubation period, 20 ml of culture medium was spun down at 12000 rpm at 4 °C for 10 min and 65 µl of each supernatant was placed in each well of an Api-Zym strip. Duplicate Api-Zym strips were used for each isolate. Intracellular enzymes had to be released from disrupted hyphae. This was done by re-suspending each pellet in 20 ml ice cold water. Samples were homogenised for 1 min using an electric homogeniser, then mixtures were returned to cold sterile centrifuge tubes and sonicated for a further 2 min to disrupt the cells and release the enzymes. Samples were kept chilled at all times to prevent enzyme denaturation. Following cell disruption, samples were spun at 10000 rpm for 15 min at 4 °C then 65 µl of each supernatant was dispensed into duplicate APi-Zym strips.

Enzyme reactions were allowed to develop for 5 hours at 37 °C. After the incubation period, 1 drop of ZYM A and 1 drop of ZYM B (BioMérieux, France) reagents were added to each of the 20 wells in the strips. Colour was allowed to develop 5 min and each strip was placed under a fluorescent desk lamp (40 W) for 10 sec to reduce background colour. Reactions were scored against the Api-Zym system's colour scale which ranged from 0 (negative) to 5 (maximum positive). 1 corresponded to 5 nMole; 2 to 10 nMole; 3 to 20

nMole; 4 to 30 nMole and 5 to 40 nMole or more, of each Api-Zym substrate metabolised by the isolates. The experiment was done on two separate occasions.

Results

Data analyses are still in progress for radial growth rates and enzyme activity studies. However, some preliminary observations can be made. For isolates grown on SEMA, the temperature optimum was found to be between 18-20°C (Table 3.8; Figure 3.7). However, there were variations amongst isolates at all temperatures. *Pandora kondoiensis* failed to grow at 25 °C whereas *P. neoaphidis* grew at this temperature. Isolate ARSEF 5374 had the slowest growth overall and NW 356 had the fastest. Some *P. neoaphidis* isolates had reasonable growth at 10-15 °C (e.g. NW 195, ARSEF 3251), as did *P. kondoiensis*. Growth was greatly reduced at 4 °C for most isolates, although not entirely inhibited, and there were exceptions which grew well at this temperature (e.g. NW 356, ARSEF 3251). A few isolates of *P. neoaphidis* had reasonable growth at 25 °C (NW 195, ARSEF 3251).

Table 3.8: Temperature optima for radial growth experiments on SEMA (data still under analysis)

Experiment no.	Isolates tested	Temp. optimum, °C
1	NW 316	20 – 22
	NW 327	18 – 20
	NW 356	18 – 20
	ARSEF 833	18 – 22
2	KVL 630	18 – 22
	NW 327	18 – 20
	NW 343	15 – 22
	ARSEF 5374	18 – 20
	NW 428	18 – 20
	NW 195	15 – 22
3	NW 283	18 – 22
	NW 314	15 - 22
	ARSEF 3251	15 - 22
	ARSEF 5708	15 - 20
	NW 415	15 - 20
	NW 416	18 - 22
4	NW 283	18 - 22
	NW 314	15 - 20
	NW 316	20 - 22
	NW 343	15 - 22
	NW 356	18 - 22
	ARSEF 5374	15 - 20
	NW 428	20 - 22
5	KVL 630	18 - 25
	ARSEF 833	18 - 20
	ARSEF 3251	15 - 22
	ARSEF 5708	15 - 20
	NW 415	15 - 18
	NW 416	18 - 25
	NW 195	15 - 22

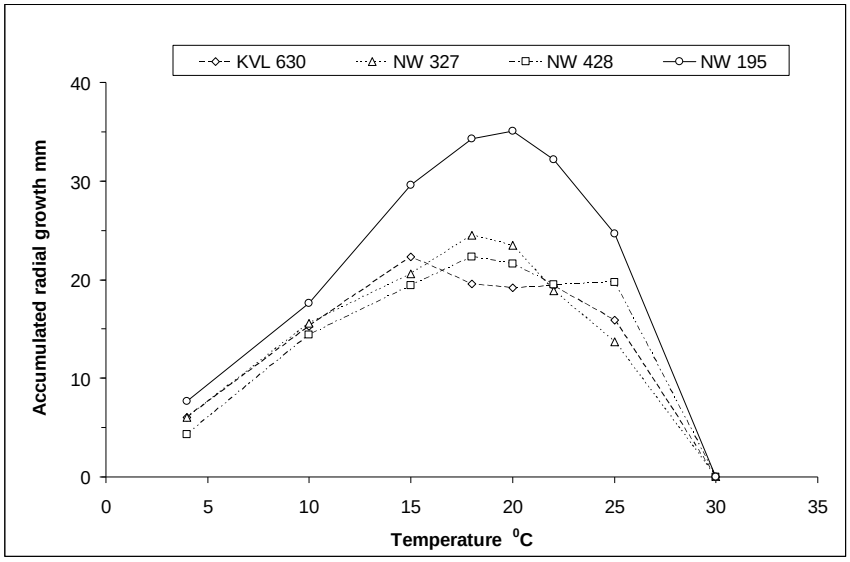

Figure 3.7: Examples of accummulated growth rates on SEMA by selected *P. neoaphidis* isolates

Results for growth rates on SDEM were mixed. Isolates separated into two main groups; those that had strong growth and those which had weak growth. *Pandora kondoiensis* had the greatest accumulated radial growth, whereas *P. neoaphidis* isolate NW 428 failed to grow (Figure 3.8).

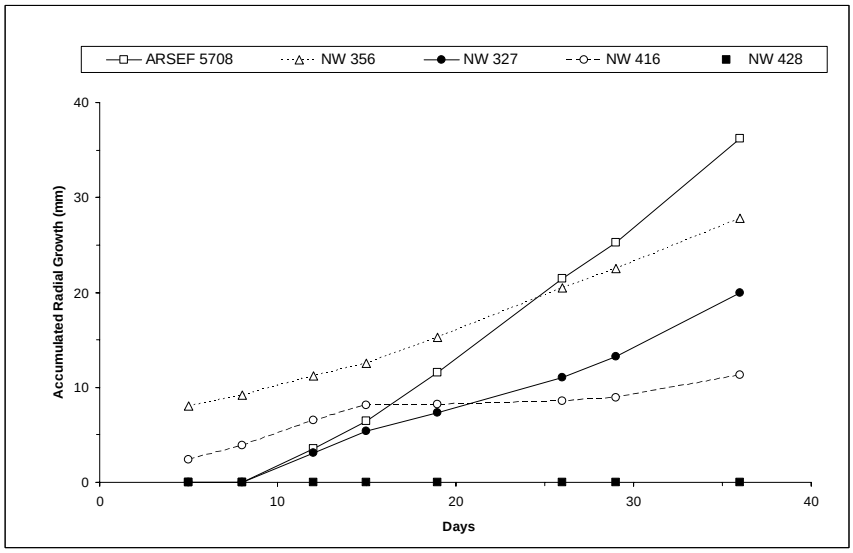

Figure 3.8: Cumulative growth on SDEM by selected *Pandora* isolates at 20°C

From Api-Zym experiments, seven extra-cellular enzymes (Nos. 4, 5, 10, 13, 14, 15 and 20) and four intra-cellular enzymes (Nos. 5, 14, 15 and 20) were not detected. Some enzymes appeared more variable between isolates (Figure 3.9). The five main extra cellular enzymes which appeared different

between isolates included leucine arylamidase (No. 6) and trypsin (No. 9). Six intracellular enzymes showing differences included esterase lipase (No. 4) and trypsin (No. 9). Enzyme analysis clearly distinguished *P. kondoiensis* from *P. neoaphidis* on the basis of absence of extra-cellular chitinase and intra-cellular galactosidase.

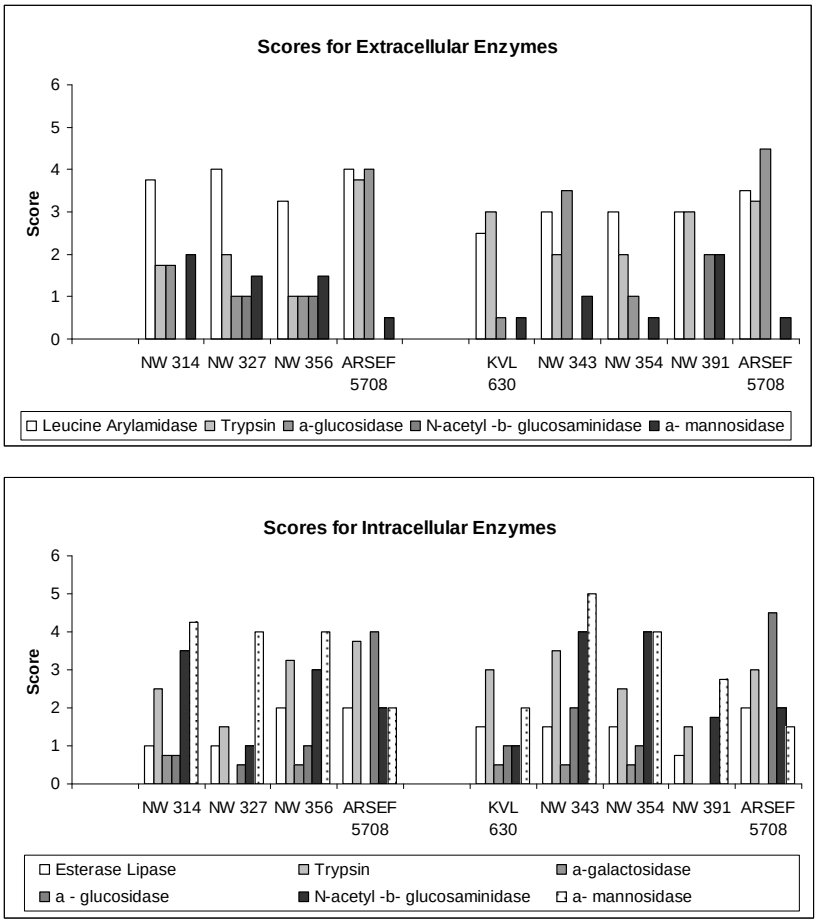

Figure 3.9: Enzyme activities for different *Pandora* isolates using Api-Zym on two different occasions

Discussion

Effects of temperature have been used to investigate the growth and virulence of a number of entomopathogenic fungi (e.g. Ouedraogo *et al.* 1997; Yeo *et al*. 2003). Previous work on *P. neoaphidis* demonstrated that germination of primary and secondary conidia was temperature dependent (Morgan *et al*. 1995). Although only one *P. kondoiensis* isolate was used, its growth at cooler temperatures and on comparitively nutrient poor media (SDEM) may indicate its greater ability to infect aphids early in the season and different host range (Tymon *et al.*, 2004). Based on speed of growth on SEMA at the optimum temperatures of 18 – 22 °C, isolates separated into those which grew very fast, those which were much slower and those which had intermediate growth rates in our studies. The majority of isolates fell into the intermediate group, with only a few at the extremes. However, this intermediate group also demonstrated variability between isolates. Many isolates were able to grow at lower than optimal temperatures. This may be an indication that season long activity of *P. neoaphidis* may occur from a succession of a series of different isolates with differing temperature optima and host virulence.

Temperature optima for most isolates on SEMA agreed with those of Morgan *et al*. (1995). The use of artificial medium may affect the germination, growth and virulence of other entomopathogenic fungi. In our study, differences between isolates were more pronounced when growth on SDEM was compared with SEMA. Several isolates, including *P. kondoiensis*, grew vigorously but others grew poorly or not at all. This result possibly reflects the ability of isolates to utilise components of the media, and may indicate differences in enzyme production required to produce the essential nutrients necessary for growth, from minimal medium. It may also reflect differences in aphid host range. Interestingly, isolate NW 356, originally from nettle aphid, grew well on both SEMA and SDEM.

Although temperature had a major effect on growth, examination of enzyme profiles may also help to distinguish between isolates. Api-Zym analysis revealed some differences between isolates in both intra- and extra-cellular enzyme production. Many of the main differences detected were with enzymes involved in carbohydrate and protein degradation. Previous work demonstrated that infection by *P. neoaphidis* altered the profile of enzymes produced by aphids (Winney, 2001), where significant differences in the production of trypsin and mannosidase between infected and uninfected aphids was demonstrated. Speed of penetration through aphid cuticle may give an indication on the virulence of an isolate. The production of chitinases, lipases and proteases, which help break down aphid cell walls, may therefore play a role in the virulence of isolates.

Host-fungus interactions in bioassays are often done under optimal conditions for fungal development. If these are not representative of the conditions which the fungus is exposed to in the field, then predictions on virulence and speed of infection may be overestimated. Further studies are required to assess isolate virulence at temperature optima obtained from this work in conjunction with studies on changes in Api-Zym profiles pre- and post-infection. Such work may improve predictions of field activity.

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Yeo H, Pell J K, Alderson PG, Clark SJ and Pye BP (2003). Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two pea aphid species. Pest Management Science, 59: 156-165.

Wilding N and Brobyn PJ (1980). Effects of fungicides on the development of *Entomophthora aphidis*. Transactions of the British Mycological Society, 75**:** 297-302.

ANNEX 1

LIST OF PUBLICATIONS, MEETINGS AND POPULAR ARTICLES

Refereed publications in scientific journals

1. Shah, P.A. and Pell, J.K. (2003) Entomopathogenic fungi as biological control agents. *Invited Mini-Review Applied Microbiology and Biotechnology* 61: 413-423
2. Shah, P.A., Clark, S.J. and Pell, J.K. (2003) Direct and indirect estimates of *Pandora neoaphidis* conidia in laboratory bioassays with aphids. *Journal of Invertebrate Pathology* 84: 145-147
3. Shah, P.A., Clark, S.J. and Pell, J.K. (2004) Assessment of aphid host range and isolate variability in *Pandora neoaphidis* (Zygomycotina: Entomophthorales). *Biological Control* 29: 90-99
4. Tymon, A.M., Shah, P.A. and Pell, J.K. (2004) PCR-based molecular discrimination of *Pandora neoaphidis* isolates from related entomopathogenic fungi and development of species-specific diagnostic primers. *Mycological Research,* 108: in press

Submitted manuscripts

1 Tymon, A.M. and Pell, J.K. Use of ISSR, ERIC and RAPD techniques to detect genetic diversity in the aphid pathogen *Pandora neoaphidis*. *Submitted to Applied and Environmental Microbiology March 2004*

2 Ekesi, S., Shah, P.A., Clark, S.J. and Pell, J.K Conservation biological control with *Pandora neoaphidis*; implications of aphid species, host plant and predator foraging. *Submitted to Agricultural and Forest Entomology March 2004.*

3 Shah, P.A., Gatehouse, A.M. R, Clark, S.J. and Pell, J.K. Wheat containing snowdrop lectin (GNA) does not affect infection of the cereal aphid *Metopolophium dirhodum* by the fungal natural enemy *Pandora neoaphidis. Submitted to Molecular Ecology March 2004*

Conference proceedings

1. Shah, P.A., Tymon, A. and Pell, J.K. (2001). Conservation biocontrol with mycopathogens: *Erynia neoaphidis* and agricultural field margins. Abstract. Proceedings of the XXXIV Annual Meeting of the Society for Invertebrate Pathology, Noordwijkerhout, The Netherlands, August 2001.
2. Tymon, A., Shah, P.A. and Pell, J.K. (2001). Assessment of various PCR based molecular techniques for developing diagnostic markers for *E. neoaphidis*. Abstract. Proceedings of the XXXIV Annual Meeting of the Society for Invertebrate Pathology, Noordwijkerhout, The Netherlands, August 2001.
3. Pell, J.K., Shah, P.A. and Tymon, A. (2002). Novel strategies for aphid control using entomopathogenic fungi. Abstract. HGCA Biennial Research and Development Conference, Coventry, January 2002
4. Shah, P.A., Clark, S.J. and Pell, J.K. (2002). A tiered approach for evaluating *Erynia neoaphidis* isolates against seven aphid species. Abstract. Proceedings of the VIIIth International Colloquium on Invertebrate Pathology and Microbial Control, VIth International Conference on *Bacillus thuringiensis* and the XXXV Annual Meeting of the Society for Invertebrate Pathology, Iguassu Falls, Brazil,

August 2002.

5. Shah, P.A., Tymon, A. and Pell, J.K. (2002). Field-scale studies on spatio-temporal relationships between aphids and natural enemies. Abstract. Proceedings of the VIIIth International Colloquium on Invertebrate Pathology and Microbial Control, VIth International Conference on *Bacillus thuringiensis* and the XXXV Annual Meeting of the Society for Invertebrate Pathology, Iguassu Falls, Brazil, August 2002.
6. Tymon, A., Shah, P.A. and Pell, J.K. (2002). Molecular studies on intra-specific variation in the aphid pathogenic fungus *Erynia (= Pandora) neoaphidis.* Abstract. Proceedings of the VIIIth International Colloquium on Invertebrate Pathology and Microbial Control, VIth International Conference on *Bacillus thuringiensis* and the XXXV Annual Meeting of the Society for Invertebrate Pathology, Iguassu Falls, Brazil, August 2002.
7. Shah, P.A., Conrad, K., Tymon, A. and Pell, J.K. (2002). Spatiotemporal analysis of aphids and natural enemies in a cereal field. British Ecological Society Annual Winter Meeting December 2002, York.
8. Pell, J.K. and Shah, P.A. (2003). Novel strategies for aphid control using entomopathogenic fungi. Proceedings Sustainable Arable LINK Conference, Rothamsted, June 2003
9. Shah, P.A. and Pell, J.K. (2003). Managed field margins as refugia for *Pandora neoaphidis*. Abstract. Invited Symposium. XXXVI Annual Meeting of the Society for Invertebrate Pathology, Burlington, Vermont, USA, July 2003.
10. Shah, P.A., Clark, S.J. and Pell, J.K. (2003). Ecological role of the large nettle aphid, *Microlophium carnosum*, as an early season source of *Pandora neoaphidis.* Abstract. XXXVI Annual Meeting of the Society for Invertebrate Pathology, Burlington, Vermont, USA, July 2003.
11. Shah, P.A., Clark, S.J. and Pell, J.K. (2003). Tritrophic interactions between *Pandora neoaphidis*, three aphid species and different host plant resources. Abstract. XXXVI Annual Meeting of the Society for Invertebrate Pathology, Burlington, Vermont, USA, July 2003.
12. Shah, P.A., Clark, S.J. and Pell, J.K (2003). Population dynamics of the large nettle aphid, *Microlophium carnosum*, and entomopathogenic fungi. Proceedings, Annual Meeting of the British Ecological Society, September 2003, Manchester, UK.

Invited talks and Presentations

Presentations were made at all HGCA monitoring between 2000 and 2003.

1. Pell, J.K. (2001). Novel strategies for aphid control using entomopathogenic fungi. Processors and Growers Research Organisation, Peterborough, Jan. 2001.
2. Pell, J.K. (2001). Where are we now with Biocontrol?. ARIA Workshop, IACR-Rothamsted, Nov. 2001
3. Shah, P.A., Tymon, A. and Pell, J.K. (2001). Conservation biocontrol with mycopathogens: *Erynia neoaphidis* and agricultural field margins. Thirteenth Meeting of the British Invertebrate Mycopathologists, Silsoe, September, 2001
4. Pell, J.K. (2002). Introduction to SAPPIO project on field margins and conservation of aphid

pathogens, FWAG Training Workshop, Barnsley, January 2002.

5. Pell, J.K. (2002). Ecology and exploitation of entomopathogenic fungi. Invited seminar, University of Staffordshire, Stoke on Trent, March 2002
6. Pell, J.K. (2002). Habitat Management for Entomophthorales. Workshop of Working Groups I and II, COST Meeting, Crete, April 2002
7. Pell, J.K. (2002). Novel strategies for aphid control using entomopathogenic fungi. Presentation at IACR-Rothamsted to DEFRA Agri-Environment Representatives. April 2002
8. Shah, P.A., Tymon, A. and Pell, J.K. (2002). Evaluation of *Erynia neoaphidis* isolates. Oral presentation, Fourteenth Meeting of the British Invertebrate Mycopathologists, Bath, September, 2002
9. Tymon, A., Shah, P.A. and Pell, J.K.(2002). Molecular studies on inter- and intra-specific variation in closely related entomophthoralean fungi. Oral presentation, Fourteenth Meeting of the British Invertebrate Mycopathologists, Bath, September, 2002
10. Pell, J.K. and Shah P.A. (2002). Microbial control using entomopathogenic fungi. Workshop, Colegio de Postgraduados, Mexico, December 2002
11. Shah, P.A. (2003). Field margins as sources of fungal diseases of aphids. Rothamsted Research Association Science Day, 20th June 2003. Rothamsted Research, Harpenden, UK.
12. Pell, J.K. (2003). Pathogen ecology and its implications for exploitation strategies under development for insect pest control. Invited seminar. Colegio de Postgraduados, Instituto de Fitosanidad, Montecillo, Texcoco, Mexico, October 2003.
13. Pell, J.K. and Shah P.A. (2003). Microbial control using entomopathogenic fungi. Workshop, Colegio de Postgraduados, Mexico, October 2003
14. Shah, P.A. (2003). Two strategies (inundation and conservation) for insect pest control with entomopathogenic fungi. 14th October 2003, Instituto de Fitosanidad, Colegio de Postgraduados, Montecillo, Mexico.
15. Shah, P.A., Ekesi, S., Clark, S.J. & Pell, J.K. (2003). Susceptibility of crop and non-crop aphids to *Pandora neoaphidis* isolates. 15th Annual Meeting of the British Invertebrate Mycopathologists Group, Sept. 24th, 2003, HRI Wellesbourne, England.
16. Pell, J.K. & Shah, P.A. (2004). Novel strategies for aphid control using entomopathogenic fungi. HDC Vegetable Growers Symposium, Wellesbourne, January 2004.
17. Shah, P.A. & Pell J.K. (2004). Field margins as refugia for *Pandora neoaphidis*. Invited lecture to students, Anglia Polytechnic University, Cambridge, February 2004.

Reports

1. Shah, P.A. (2001). Short-term Scientific Mission to Poland, Sept. 9th-23rd 2001. Report for EU COST 842.
2. Chan, E.and Shah, P.A. (2002). Use of the polyphenol oxidase enzyme assay to assess resistance of aphids against the entomopathogenic fungus *Erynia neoaphidis.* HGCA report for student bursary under SAPPIO LINK 1159 "Novel strategies for aphid control using entomopathogenic fungi".

Technology transfer events

- HGCA Cereals events, attendance at R&D stands in 1999, 2002 and 2003
- FWAG workshop in 2002
- Processors and Growers Research Organisation in 2001
- HGCA R&D meetings in 2002 and 2004
- HDC Vegetable Growers Symposium in 2004

Articles for industry stakeholders

- "Fungi for Controlling Aphids" SAPPIO Programme Factsheet, 2001
- "Pathogenic Fungi: Natural Borne Aphid Killers" *agriculture*link, February 2001
- "Field margins can encourage aphid pathogens" Arable Farming, August 18th 2001
- "Fungi for controlling aphids" Pea and Bean Progress, Spring 2002
- "Field margins and fungal diseases of aphids" Farming and Wildlife Advisory Group website under "New Research Directions & Diversification" (http://www.fwag.org.uk/)
- "Fungi for aphid control" St. Albans Observer, 2001