

Improving secondary pick up of insect fungal pathogen conidia by manipulating host behaviour

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Summary

It is often assumed that efficient application of a mycoinsecticide involves hitting the target pest insect directly with a lethal dose of conidia. However, secondary pick-up of conidia from surrounding vegetation may be a more important source of inoculum. We have investigated ways of increasing conidia acquisition by enhancing host movement. The aphid alarm pheromone, E- β -farnesene, significantly increased mortality among peach potato aphids, *Myzus persicae* Sulzer, that were exposed for 24 h to discs of green pepper leaf sprayed with conidia of *Verticillium lecanii* (Zimmerman) Viegas then transferred to fresh untreated discs to allow disease development. A more practical approach to increasing conidia pick-up appears to be the use of sub-lethal doses of the chloronicotiny insecticide imidacloprid. One percent of the recommended dose, applied systemically, dramatically increased aphid movement; quantified by image analysis of videotaped aphid behaviour. This resulted in greater mortality from mycosis in experiments where aphids were exposed to insecticide-treated leaf discs that had been sprayed with fungal conidia. A comparison with results from an experiment where conidia were sprayed directly onto aphids which were feeding on insecticide-infused pepper discs established that synergy was due to an indirect effect of the insecticide, i.e. through increased movement, rather than a direct effect *viz.* predisposition of insecticide-weakened insects to disease.

Key words: Aphids, *Myzus persicae*, *Verticillium lecanii*, secondary pick up, imidacloprid, alarm pheromone, fungal conidia

Introduction

Fungal pathogens have potential for control of insect pests. So called mycoinsecticides are being used on a limited scale for example against scarab beetles on sugar cane in Australia, whiteflies on glass house crops in Europe and grasshopper and locusts in Africa (see review by Charnley, 1997). The ability of fungi to penetrate actively host external skeleton denotes contact activity; other microbial pathogens have to be ingested. This important attribute has, however, suggested the importance of hitting the target. Recent work, in particular on locusts, has demonstrated that direct impact may be less important than secondary pick up from vegetation (Thomas, Wood, Langewald & Lomer, 1997). In the light of this, we have been looking at ways of increasing insect movement in order to optimise pick-up of conidia from a sprayed surface. Our experiments have employed the interaction between the Ascomycete *Verticillium lecanii* (Zimmerman) Viegas and the peach potato aphid *Myzus persicae* Sulzer.

Aphids are known to produce alarm pheromone, released from cornicle secretions for example when an individual is attacked by predators or parasitoids. Neighbouring aphids respond to alarm pheromone

with defensive or avoidance behaviour such as dispersing or dropping off the leaf. The main chemical component of the pheromone of most aphid species is (E)- β -farnesene (EBF) (Pickett, Wadhams, Woodcock & Hardie, 1992). Increasing aphid mobility with EBF improves pick up of pesticides (Griffiths & Pickett, 1980, 1987) and control of the cotton aphid *Aphis gossypii* Glover by the fungal pathogen *V. lecanii* (Griffiths & Pickett, 1987). In the latter case it was assumed that increased movement of aphids resulted in enhanced pick up of conidia, but this remains to be confirmed.

Another approach to increasing secondary conidia pick-up is to employ sub-lethal doses of a chemical insecticide that has antifeedant or other irritant properties. The chloronicotiny insecticide imidacloprid has these characteristics. Imidacloprid is particularly effective against homopteran pests such as aphids, leafhoppers and whiteflies (Elbert *et al.*, 1996; Nauen *et al.*, 1996). Aphids, feeding on plant tissue infused with sublethal doses of imidacloprid solution, showed decrease in weight, depression of honeydew excretion and restless behaviour. Ultimately aphid death from starvation occurred if the insects were left on the treated tissue. When the aphids were transferred to untreated leaves, they recovered well.

The reversible starvation response was therefore consistent with an antifeedant effect rather than symptoms of neuronal disorder (Nauen, 1995).

Use of chemicals as 'stressors' to enhance the efficacy of mycopathogens has been proposed many times (e.g. Anderson *et al.*, 1989; Hassan & Charnley, 1989; Hassan, Dillon & Charnley, 1989; Quintela & McCoy, 1998a). The synergism manifests itself in mortality levels elevated above those obtained following exposure to the insecticide or the fungus alone (Gardner & Kinard, 1998). Boucias, Stokes, Storey & Pendland (1996) showed synergism between imidacloprid and the fungus *Beauveria bassiana* (Balsamo) Vuillemin in termites. This interaction proved to be due to insecticide-induced disturbed grooming behaviour and other social activities common in termites; absence of these hygiene functions increased levels of mycosis. Quintela & McCoy (1997, 1998b) indicated that reduced larval mobility and associated conidial avoidance was the basis of synergism of imidacloprid with both *Metarhizium anisopliae* Sorok and *B. bassiana* treated larvae of root weevil *Diaprepes abbreviatus* Linnaeus. The present work has established the converse situation, namely that increased movement of *M. persicae* brought about by sub-lethal doses of imidacloprid and EBF promote pick-up of *V. lecanii* conidia.

Materials and Methods

The aphid alarm pheromone, (E)- β -farnesene, (61% pure in hexane) was provided in glass ampoules by Rothamsted Experimental Station. Imidacloprid of technical purity (> 95%) was used. All experiments were carried out using the *M. persicae* clone R1 (405D) (provided by Rothamsted Experimental Station). Large numbers of aphids were reared on potted green pepper plants *Capsicum annuum*, cv. California Wonder (Moles Seeds) in muslin covered wooden cages (1 m³). Cages were kept in an air-conditioned room at 21 \pm 1°C and a photoperiod of 16 h light (L): 8 h dark (D). Stock cultures of each aphid clone were maintained on small excised leaves according to the method of Blackman (1988). The isolate KV71 of *V. lecanii* (active constituent of the mycoinsecticide Vertalec) was provided by Koppert B.V. Bactopeptone Agar (2% (w/v) Malt extract (Oxoid), 5% (w/v) Bacteria peptone (Difco Laboratories) and 2% (w/v) Agar (Agar No. 3, Oxoid)) was used for routine fungal culture. Cultures were kept at 24°C and harvested after 8 days in 10 ml of sterile distilled water (dH₂O).

Effect of alarm pheromone on secondary pick up

1.7 ml of molten sterile water agar (1% (w/v) agar in dH₂O) was poured into each of five compartments of 25-compartment sterile square repli dishes. As

soon as the water agar cooled down a square piece of pepper plant leaf, specially cut to fit the area of each compartment, was placed on the agar with the abaxial surface uppermost. Fluon[®] was applied on the edge of each compartment, to prevent the aphids from climbing on the compartment's walls and escaping. The dish was sprayed with 1.5 ml sterile dH₂O (control), 10⁶, 10⁷ or 10⁸ *V. lecanii* conidia per ml using a Potter tower (Burkard) then allowed to dry for 15 min. Approximately five aphids were placed in each compartment and left to settle for 2-3 h. 1 μ l of EBF (in hexane) was introduced via a microsyringe onto small pieces of filter paper (diameter 2 mm) into the centre of the sprayed compartment. 1 μ l of hexane was used as a control. The compartments were immediately sealed with Parafilm M[®] and two holes were made above each compartment for ventilation. All repli dishes were incubated at 23 \pm 1°C with photoperiod 16 h L: 8 h D. After 24 h the aphids were transferred to clean individual chambers for a further 6 day incubation (temperature 23 \pm 1°C, 16 h L: 8 h D). For the first three days the replidishes were left alone to ensure 100% r.h. during a period long enough to allow infection to occur. Plates were opened daily from days 4 to 7 and dead or missing insects were recorded. An aphid was considered dead from *V. lecanii* when the characteristic white-coloured sporulation on the insect's body was observed in combination with inability to walk and absence of response when the insect was prodded with a fine seeker. In case of doubt of the cause of death, the aphid was carefully examined under a dissection microscope.

Effect of imidacloprid on aphid behaviour

A tracking procedure was developed to study the effects of sublethal systemic doses of imidacloprid on aphid behaviour. The procedure comprised three successive phases; 1) filming of aphid activities on videotape, 2) computerised videotape analysis (data extraction) and 3) data analysis.

Filming set up and preparation of the leaf disks

The equipment was placed in an incubator with constant temperature of 24°C and photoperiod at 16 h L: 8 h D. The camera used was a Panasonic NV-SX30B. The stand was placed on cork to absorb any vibrations caused from the motor of the incubator. Six fluorescence tubes (GE, F8W/35) were placed a few cm above the arena providing illumination of 1200 Lux. The aphids were filmed for 12 h from the time that they were introduced to the leaf surface of the filming arena.

The arena was a 5 cm Petri dish (deep) with 6 ml of 1% water agar (w/v) and a leaf disk placed on top. Imidacloprid was systemically applied to pepper leaves by infusion prior to preparation of the leaf discs. Imidacloprid stock solutions were prepared in

acetone (1 mg of imidacloprid/0.5 ml acetone) and were subsequently diluted to the appropriate concentration with distilled water. The final dilution, used to infuse the leaves, was made up with tap water. Pepper leaves were cut and their petioles immediately immersed in 0.1 ppm of imidacloprid (sublethal dose determined by E Siskos and S E Reynolds, personal communication) or water for the control treatment. After allowing 24 h for infusion, leaf disks (5 cm diameter) were cut and filming arenas were prepared. Five adult aphids were placed in the arena, which was then placed under the camera. Time-lapse filming started once the image was centred and focused (12 h of real time in 3 h of videotape). The analysis of the 12 h was split into three periods each 4-h long. The behaviour of 25 aphids was analysed per treatment (five replicates of five aphids each).

Computerised analysis of video tapes and data extraction

An outline of the two-step method is described here as a full description is given elsewhere (Couzin, 1999; Roditakis, 1999). In the first step a computer was connected to the VCR through a Matrox Meteor frame grabbing computer board. The analogue image of the videotape was digitised to a 768×576 -pixel greyscale image. An aphid tracking program was developed and its basic function was continuous analysis of digitised images and the identification and location of the aphids on the images using blob analysis. The program recorded the number of blobs, their position on the image (x,y co-ordinates of the centre of gravity of the blob) and the time that the image was captured from the initiation of the analysis session on formatted text files (data arrays). Around six frames per second were analysed in a typical session.

In the second step it was possible to mark each blob with a unique numeric identifier, representing an individual aphid. An array of sequential frames with the same identifier could then be extracted (formatted text file). Each array represented the sequential positions of a particular aphid on the arena with the time. In this way the path followed by an aphid could be interpolated.

Data analysis

The data were processed by Microsoft Excel and the distance covered was calculated using simple geometry.

Effects of imidacloprid on secondary pick up

Discs were cut of leaves infused in 0.1 ppm imidacloprid water solution ('Imidacloprid' treatment) or water ('Water' treatment) for 24 h and placed on 6 ml of 1% (w/v) water agar in a 5 cm Petri dish. Twelve Petri dishes were used for each treatment; six fungus inoculated dishes (sprayed with

1.5 ml conidia suspension of 2.6×10^6 conidia ml^{-1}), and six control dishes (sprayed with 1.5 ml sterile dH_2O). The Petri dishes were left to dry for 15 min. Then eight to 10 aphids were applied onto each dish and were left to feed on the leaf surface for 24 h. After that time interval the aphids were transferred to individual uninfected chambers in repli dishes. Mortality was determined after a 7-day incubation ($23 \pm 1^\circ\text{C}$, 16 h L: 8 h D).

Investigating synergism between *V. lecanii* and imidacloprid

Five cm Petri dishes were prepared as described in the previous experiment using 0.1 ppm imidacloprid or water treated leaf disks. Twelve dishes were prepared for each treatment (six to be inoculated, six control). 8-10 aphids were transferred to each dish and were left to feed on the leaf discs for 24 h, as in the previous experiment. After the 24-h period the aphids were sprayed directly with 1.5 ml of conidia suspension (2.1×10^6 conidia ml^{-1}) or sterile dH_2O (control treatment). The aphids were transferred to uninfected repli dishes, after allowing the sprayed suspension to evaporate (10 min). Mortality was assessed after 7 days of incubation ($23 \pm 1^\circ\text{C}$, 16 h L: 8 h D).

Results

Effect of alarm pheromone on secondary pick up

Aphids were placed for 24 h on the leaf surface sprayed with conidia. After the first 2-3 h (settling period) the aphids were exposed to EBF, hexane or left untreated. Subsequently insects were transferred to a non-contaminated environment to allow mycosis to develop. Mortality among aphids exposed to EBF was significantly greater than either hexane or

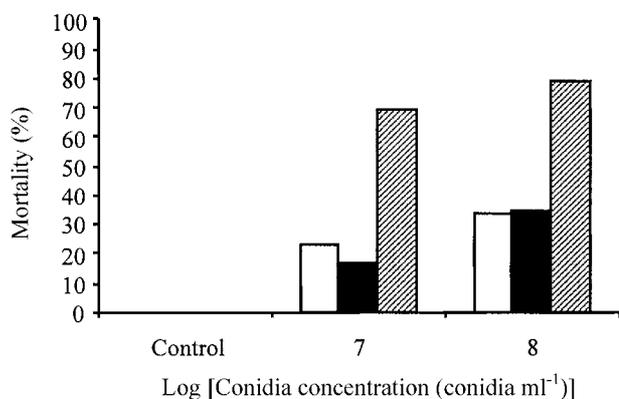


Fig. 1. Aphid mortality by secondary pick up of *V. lecanii* conidia for insects exposed to the alarm pheromone. Combined mortality recorded from three experiments (25 aphids per treatment). Treatments; 'Hexane' = solvent (hexane) alone, 'EBF' = alarm pheromone in hexane, control = untreated. □ Untreated; ■ Hexane; ▨ EBF.

Table 1. Statistical analysis of the effect of EBF on the aphid mortality in 'secondary pick up' experiments (see Fig. 1). χ^2 test was applied for comparison between treatments Untreated, Hexane and EBF. Treatments within a column followed by the same letter are not significantly different ($P < 0.05$), $N = 25$ for each treatment

Treatment		Applied Conidia Suspension (conidia ml ⁻¹)							
		Control		10 ⁶		10 ⁷		10 ⁸	
Water	Total dead	0	a	8	ab	14	a	19	a
	Total alive	25		16		10		5	
Hexane	Total dead	0	a	5	a	13	a	17	a
	Total alive	25		17		11		7	
EBF	Total dead	0	a	12	b	13	a	20	a
	Total alive	25		12		12		4	

untreated controls, at both conidia concentrations (see Fig. 1 and Table 1).

Effects of imidacloprid on aphid behaviour

The path followed by three individual aphids feeding on water (control) or imidacloprid treated leaves during the 12 h filming period is displayed in Fig. 2. After the first 4 to 8 h of searching behaviour all three aphids became settled in the control treatment, while aphids on imidacloprid infused leaves failed to find a feeding site and continued to move.

The distance covered by the aphids was calculated for each of the 3 periods (Fig. 3). The data were not normally distributed, hence the median is used to describe the data. The aphids on control (water) treated leaves showed mobility only in the first section of the 12 h period. The median distance covered was 344 mm in the first 4 h and 0 mm in both the following 4 h periods (4-8 and 8-12 h). In comparison aphids feeding on imidacloprid treated leaves were mobile throughout the 12 h filming period. The distance covered ranged between 280 to 460 mm over the 4 h. Significant differences were detected between treatments for the later filming periods, 4-8 and 8-12 h (Table 2).

Effects of imidacloprid on secondary pick up

This experiment was designed to study the impact of the behavioural disturbance of aphids, on imidacloprid treated leaves, on the secondary pick up of conidia from the leaf surface. The mortality recorded for the treatments after the 7-day incubation period is displayed in Fig. 4A. Insects exposed to imidacloprid treated leaves infected with *V. lecanii* showed significantly greater mortality than insects on water treated leaves.

Investigating the case of synergism between *V. lecanii* and imidacloprid

Increased mortality among aphids on imidacloprid treated leaves could be due to the detrimental effects of the insecticide on the insect rather than enhanced

pick up of conidia. The possibility that the insecticide and fungus can act synergistically was investigated as follows.

Aphids were left to feed for 24 h on water or imidacloprid treated leaves, then were sprayed *in situ* with *V. lecanii* conidia suspension. The mortality after a 7-day incubation is illustrated in Fig. 4B. There was no control mortality and the difference between treatments was not significant.

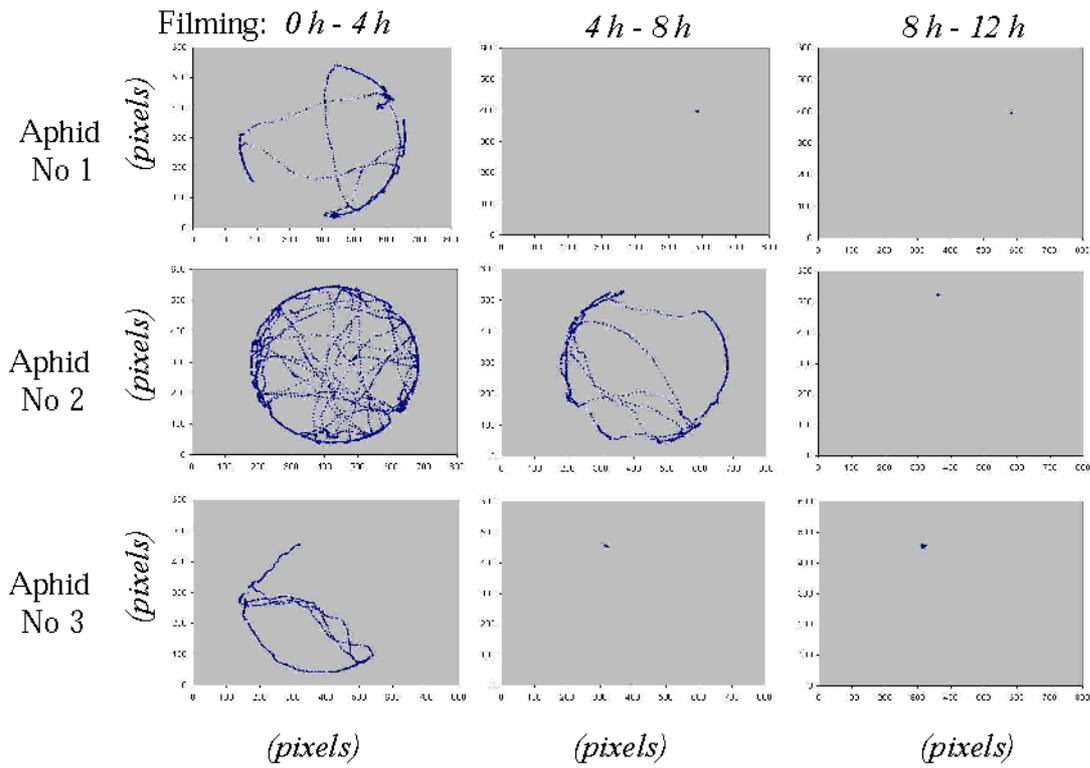
Discussion

Exposure to EBF significantly increased mortality among aphids in a situation where acquisition of a lethal dose of conidia could only occur from the leaf surface. This is the first critical laboratory experimental evidence for enhanced secondary pick up in response to EBF. The results are consistent with field trials conducted with *A. gossypii* by Griffiths & Pickett (1980, 1987), which showed enhanced aphid mortality when EBF was applied with chemical pesticide or *V. lecanii*.

It is almost three decades now since EBF was first discovered, yet no use has been found for it in insect management. Biological and chemical constraints including problems in handling, storing and applying a volatile, unstable compound have prevented its practical use (see e.g. Dawson, Griffiths & Pickett, 1984; Montgomery & Nault, 1977; Dawson, Griffiths, Pickett & Woodcock, 1983; Gibson & Rice, 1988; Wohlers, 1981). Thus while the experiments described in the present work establish the principle that increased movement of aphids can enhance conidia acquisition, combining EBF and fungus is probably not a practical option.

Sub-lethal doses of imidacloprid may prove a viable alternative. Aphids showed a significant increase in movement on insecticide-infused pepper leaves. Indeed aphids on treated leaves demonstrated continuous mobility throughout the 12 h long recording compared to aphids on control (water) treated leaves that settled in the first 4 h. The presence of imidacloprid inhibited settling and subsequent

12 hour filming - No treatment (control)



12 hour filming - 0.1 ppm *Imidacloprid*

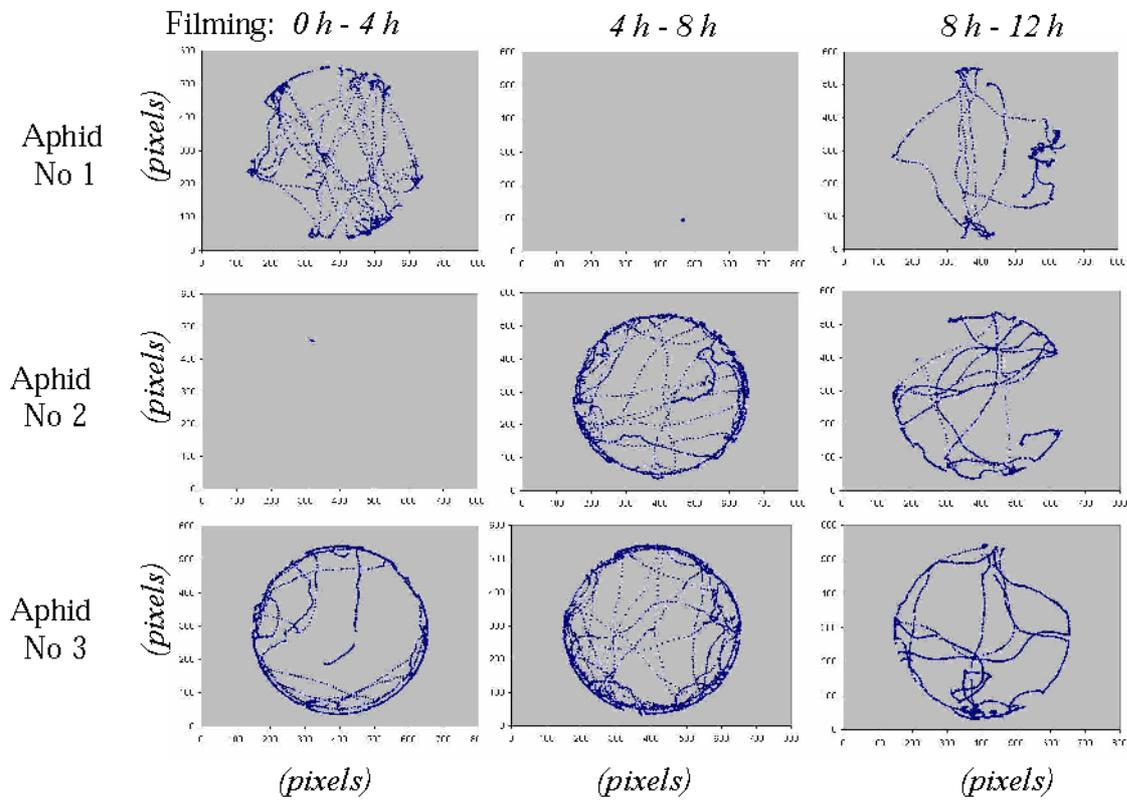


Fig. 2. Tracks of individual aphids feeding on water treated leaves (control) and on imidacloprid treated leaves in a 12 h filming period. When a single dot is observed, the aphid did not move from the feeding position. Note that all three aphids were immobile in the third section (8-12 h) of the control treatments, in contrast to the imidacloprid treatment.

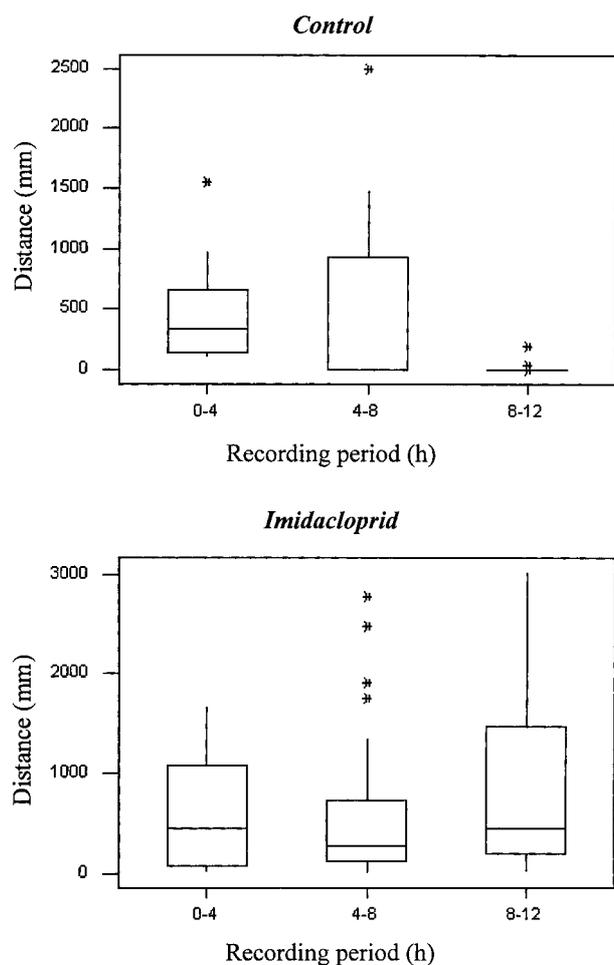


Fig. 3. Distance covered by aphids feeding on control (water) or imidacloprid treated leaves (data from five replicates, five adults each). The data are not normally distributed and therefore represented by box plots. The line dividing each box represents the data median and the interquartile range lies between the top and bottom box lines. Symbols above boxes denote outlying values that are 1.5-3 times away from the middle 50% of the data. The 12 h recording period is divided into sections of 4 h each.

feeding; this forced the insects into searching behaviour for an acceptable energy resource. Similar symptoms of restlessness and irritability were recorded by Nauen (1995), and Nauen, Tietjen, Wagner & Elbert (1998) who established that imidacloprid

Table 2. Statistical analysis of the distance covered by aphids in feeding on control (water) or imidacloprid treated leaves in the three sections of the 12 h recording. The median distance covered in (mm) is displayed. The Mann-Whitney U-test for non-parametric data was applied. Data within a column followed by different letters are significantly different ($P < 0.05$)

Treatment	Recording period (h)		
	0 - 4	4 - 8	8 - 12
Control	344.3 a	0.0 a	0.0 a
Imidacloprid	459.6 a	277.1 b	455.7 b

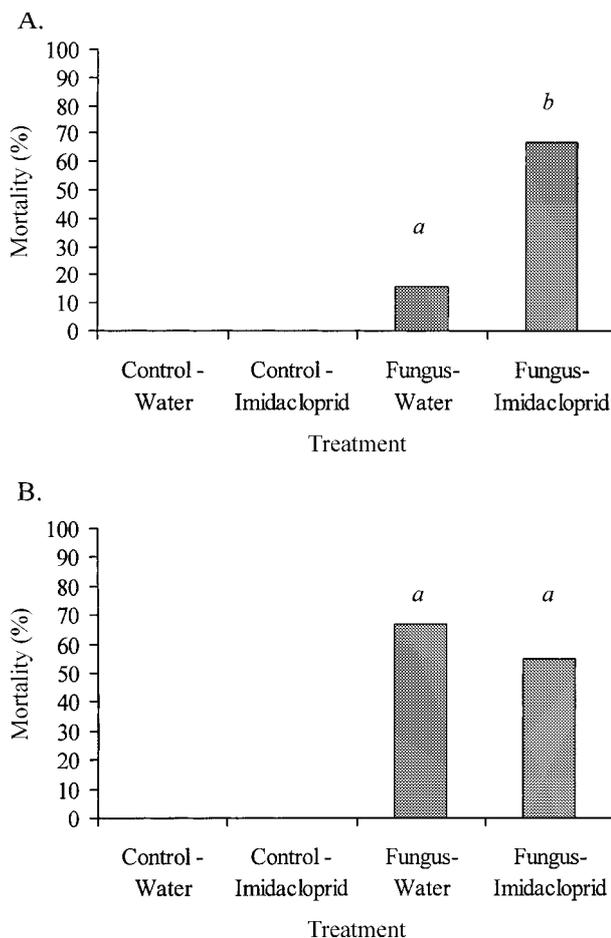


Fig. 4. Aphid mortality from *V. lecanii* following exposure to imidacloprid. Treatments with different letters are significantly different (χ^2 test was applied to the raw data, $P < 0.05$, $n = 50$ aphids per treatment). A: secondary pick up over a 24 h period of *V. lecanii* conidia from leaves infused with water or 0.1 ppm imidacloprid solution. Aphids were transferred to a fresh leaf disc and mortality recorded after a further 6 days. B: Mortality among aphids sprayed *in situ* with fungus while they were feeding on discs of pepper leaf that had come from leaves infused with imidacloprid or water. Mortality (%) recorded after 6-day incubation.

and its metabolites *in planta* have high oral antifeedant activity against *M. persicae* (Nauen, 1995; Nauen *et al.*, 1998).

Significantly increased mortality occurred among those aphids allowed to pick up conidia from imidacloprid treated leaves compared to water treated leaves. High mobility of the insects on the treated surface appears to account for the increased aphid mortality, since no synergy was found between topically applied conidia and systemic sublethal dose of imidacloprid.

This is not the first time that a clear unambiguous link has been made between increased movement and enhanced secondary pick-up. Furlong & Pell (1996), for example, observed increased levels of infection of the Diamondback moth larvae, *Plutella xylostella* Linnaeus by the entomopathogenic fungus

Zoophthora radicans Brefeld in the presence of the parasitoid *Diadegma semiclausum* Hellén. They suggested that the greater movement (as in distance covered and new areas visited) of the disturbed larvae accounted for increased inoculum pick up.

Cost and compound stability are major factors in the choice of a compound to increase aphid movement. Imidacloprid has been on the market for a number of years. Systemic applications of imidacloprid are accomplished easily by watering plants with insecticide solution. Imidacloprid has lasting effects since its metabolites themselves have high oral antifeedant activity against aphids (Nauen *et al.*, 1998). The results from the laboratory experiments described here suggest that systemic applications of the insecticide with concentration of just 1% of the recommended dose could dramatically improve the efficiency of *V. lecanii* control of aphids. Additionally, the combined use of control agents with different modes of action reduces the likelihood of simultaneous development of resistance to the two agents (Georghiou, 1994).

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