Session 12D

The Use of Beneficial Organisms in Plant Protection

Population Level Management

Chairman & Session Organiser:	Dr Gavin Lewis *JSC International, Harrogate, UK*
Platform Papers:	12D-1 to 12D-4
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Pesticides and beneficial insects: rediscovering the origins and purpose of IPM

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Introduction

Concerns about pesticide impact on the predators and parasites of crop pests were expressed in the earliest stages of development for modern synthetic pesticides. Research by the pesticide industry revealed insect pest outbreaks following applications of broad spectrum insecticides that were correlated with the elimination of predatory insects. Over the next 30 years, evidence for pest resurgence and its importance as a driver for the pesticide treadmill increased, along with a large body of research literature on the toxicity of pesticides to beneficial insects. Integrated pest management (IPM) emerged as a tool for the management of pest control tactics that had the potential to limit these adverse impacts by enabling pesticides to be used selectively, or even avoided altogether.

There are many examples of effective IPM programs, but also continuing evidence that pesticide impacts promote pest outbreaks and unnecessary pesticide applications world-wide. Efforts to develop pesticide toxicity databases and procedures for the measurement of pesticide toxicity to beneficial insects have been impressive, but attempts to develop practical IPM programs that exploit these data on a wide scale have been defeated by the complexity of pest: beneficial insect interactions within and between the agricultural systems of the world.

There has also been an increase in the degree of regulatory scrutiny that is applied to pesticide: natural enemy impacts, and tiered risk assessment procedures are still evolving that inform the regulatory process and impact the labelling and use of certain insecticides in certain jurisdictions, particularly within the European Union.

There is now an opportunity to integrate ideas from regulatory toxicology and IPM to help refine procedures that can be used to exploit the beneficial properties of pesticides while enabling pest limitation by predatory and parasitic invertebrates to continue. A concerted effort will be needed for this process to be sufficiently international in scope to benefit IPM programs in the crops that are most sensitive to disruption and impairment of biological pest suppression by pesticides.

A procedure for the applications of eco-toxicological approaches to the management of pesticide-natural enemy interactions within IPM programs is given below (adapted from Jepson, 2007). This outline assumes that exhaustive efforts have been undertaken to exploit alternatives to pesticides before these steps are undertaken.

Step 1. Analysis and management of short-term pesticide impacts

- Develop an inventory of natural enemies, including taxonomic composition, pest associations, phenology and patterns of distribution within the crop canopy and surrounding off-crop habitats.
- Develop an inventory of pesticides used against key pests, including details o application rates, application method and timing.

- Obtain literature data on susceptibility of the natural enemies that have been listed to the pesticides that they may be exposed to.
- Develop and implement a program of bioassays to confirm toxicity values from the literature, fill knowledge gaps in natural enemy susceptibility and provide toxicological statistics for risk analysis.
- Measure pesticide distribution through the crop canopy at different growth stages, using appropriate application techniques, and/or measure exposure of natural enemies to pesticides directly.
- Undertake *in-situ* bioassays to determine the persistence of toxicity of the main pesticides to key natural enemies.
- Calculate short-term risk from exposure and susceptibility data for key natural enemies exposed to the pesticides that are used when they are active in the crop canopy, and at the appropriate crop growth stages.
- Exploit opportunities for physiological selectivity by ranking pesticides in terms of toxicity to key natural enemies, and include this analysis in procedures to select pesticides for use with the IPM program.

Step 2. Mitigation of short-term risks for pesticides that are toxic to natural enemies

- Exploit ecological selectivity by avoiding application of toxic pesticides at times when key natural enemies are active, including periods in the day when they are most exposed to pesticides, and periods in the season when they are most abundant
- Exploit ecological selectivity by improved targeting of sprays to reduce natural enemy exposure, or by strip treatments

Step 3. Management of long-term risks of pesticides that are toxic to natural enemies

- Rank natural enemies in terms of the ecological attributes that underlie susceptibility to long-term ecological impacts
- Undertake field experiments that directly measure rates and patterns of recovery following chemical exposure
- Determine the mechanisms that enable recovery, including reproduction and colonization and identify sources or reservoirs of natural enemies within the agro-ecosystem
- If recovery for at-risk species takes place within the cropping season, and is considered sufficient to maintain natural enemy populations, initiate a program of monitoring that is sensitive enough to detect declines in the abundance of at-risk species between seasons
- If recovery does not take place within the cropping season, and is not considered sufficient to maintain the natural enemy population, consider expansion of natural enemy refugia in off-crop areas to enhance recovery rates
- If natural enemy recovery rates are inadequate and can not be enhanced within the cropping system when a given pesticide is in use, return to the start of the process and select alternative pesticides

Reference

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Pesticide regulation, beneficial arthropods and Integrated Pest Management

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Use of biological control in Great Britain

Biological control agents (BCA's) are widely used in UK protected horticulture. They are a predominant means of pest control in some protected crops with *Encarsia formosa*, *Phytoseilus persimilis*, *Aphidius colemani* and *Amblyseius* spp. being the most significant introductions. In outdoor horticulture, usage of BCA's is minimal although in orchard situations, *Typhlodromus pyri* is acknowledged as an effective mite predator enabling a reduction in use of acaricides to control fruit tree red spider mite.

Whether a grower is introducing a parasite or predator targeting a specific pest problem within a crop or anticipating a controlling effect from naturally occurring parasites and predators, he needs to be aware of potential adverse effects from the application of plant protection products for control of other harmful organisms, e.g. diseases. Serious adverse effects on specific parasites, predators or pathogens might disrupt the balance and result in a need for further pesticidal intervention. Information on the specific effects of pesticides in Integrated Pest Management (IPM) is therefore necessary to enable an informed choice of pesticide by the grower.

Considerable information is available to users from a variety of sources on the effects of different pesticides on a wide range of natural and introduced beneficial species. However, PSD has a policy on labeling in relation to pesticide compatibility with IPM in order to help achieve optimum use of pesticides.

Regulatory approach

Ecotoxicological risk management strategies for protection of non-target arthropods results in labeling where data indicate a risk to non-target arthropods. It is considered that this labeling is sufficient to alert practitioners of IPM that adverse effects on beneficial species may be expected and that specific warnings regarding compatibility with IPM are not required.

However pesticide product labels may carry positive statements or claims regarding compatibility of the product with parasites, predators and pathogens present naturally or introduced as biological control agents. The wording of any statement is at the discretion of the applicant, but data are required to support such statements, with data from specific studies (both laboratory and field, and published work) and from observations made during the efficacy studies being used to substantiate the claim. The nature of claims made varies considerably, but with very broad claims (e.g. compatible with IPM) being difficult to substantiate, the regulatory process typically results in specific or targeted claims relating to named beneficial species appearing on product labels. For example, 'When used as directed this product does not have adverse effects on the predatory mite *Typhlodromus pyri* or the common flower bug *Anthocoris nemorum*'

It is important that the studies conducted use the product in question at the recommended dose in the relevant situation such that exposure is representative of that to which the organism will be subjected. As such observations in efficacy studies can provide valuable

confirmation of data from laboratory or preliminary studies. Even where data do demonstrate some adverse effects valuable information for the grower may be made in the form of carefully constructed label claims; for example 'This product can have adverse effects on beneficials hit. Once dried, the product has no residual effects so they can be safely introduced into the treated area'. Typically the company will propose a suitable claim for the product label, and upon evaluation of the data, PSD will either consider the claim acceptable or may amend the claim in line with the data.

IPM and the European dimension

IPM is seen as having an important role to play in reducing the risks associated with the use of plant protection products. However at present there is no common understanding of IPM and no legally binding definition at Community level. Different public and private systems co-exist across the Community and within Member States.

Establishing such a definition and ensuring users adopt IPM are key components of the proposed EU Directive on the Sustainable Use of Pesticides (COM(2006) 373 final). This Directive is one of the key measures (along with the revision to Directive 914/414/EEC and a pesticide statistics regulation) being proposed as part of the EU Thematic Strategy for Pesticides. At the time of writing (July 2007) the proposal envisages Community-wide standards of IPM being developed and becoming mandatory from 2014. Crop specific standards will also be developed at Community level but their implementation will remain voluntary.

The European Commission's Thematic Strategy Impact Assessment estimates that adoption of the measures outlined above could lead to a 10% reduction in the use of plant protection products across the EU (it should, however, be stressed that use reduction *per se*, is not a stated aim of the Strategy).

The use of IPM is identified by the Commission's impact assessment as assisting users through: comparable or slightly higher produce prices; and reduced financial efforts buying pesticides. This will more than offset increased costs due to: comparable, or slightly lower, yields; certification requirements; comparable or slightly higher costs for buying crop varieties; and management, education and advisory services.

Conclusions

The use of biological control agents form an important component of crop protection in the UK, especially in protected horticultural crops. PSD operates a policy on labeling in relation to pesticide compatibility with IPM. This policy permits information to be presented on product labels to inform users of the expected compatibility of an application of a product with relevant biological controls agents. Claims made must be supported by data which are evaluated as part of the regulatory process.

IPM is viewed by the European Commission as having a significant role to play in reducing the risk associated with the use of plant protection products and is an important part of the proposed Sustainable Use Directive.

Effects of biological control agents (BCAs) on the pollinator, *Bombus terrestris*

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Introduction

Insects such as honeybees and bumblebees are important pollinators of wild flowers and important crop plants worldwide (Corbet *et al*, 1991; Goulson, 2003) The bumblebee *Bombus terrestris* L.is widely used in modern agriculture/horticulture to guarantee the pollination of vegetables like tomatoes and peppers, and fruits like strawberries, apples and pears, as this natural pollination results in higher fruit quality and quantity. This concept of natural pollination fits excellently within the concept of integrated pest management (IPM) with the employment of biological control agents (BCAs). However, as these BCAs products are sprayed on the leaves and the flowers, the bumblebees are exposed to the microorganisms during foraging and via the flowers. Hence, as is also the case for chemical pesticide use, the application of BCAs (e.g. biological insecticides such as *Beauveria bassiana* against pest insects and antagonists such as *Trichoderma* spp. against plant pathogens) implies the need to determine their potential risks against the pollinators in order to maintain pollination and production. To date, however, only a few studies have been performed on the possible adverse effects of such BCAs against bumblebees. Also, the data generated has been limited to acute toxicity and no information has been presented on sublethal effects reducing the bumblebee colony and the pollination. However, assessment of the risks from BCA acute toxicity and also on their potential sublethal effects is essential before any new control strategy can be recommended for use.

The objective of this research was to investigate the compatibility of four biofungicides and three biological insecticides with the pollinator *B. terrestris*. Bumblebee workers were treated under laboratory conditions with the BCAs at their respective recommended concentrations for field use (MFRC), and exposed via the three potential routes of exposure: dermal contact and orally via the drinking of sugar water and via pollen.

Material and methods

Bumblebees: For the experiments artificial nests were used, each containing five worker bees of *B. terrestris*, as described recently (Mommaerts *et al*, 2006a & b). The artificial nests are produced in-house and made of transparent plastic (15 cm x 15 cm x 10 cm) and kept under standardized laboratory conditions in the dark at 30±2°C and 60±10% r.h.

BCAs tested: The seven BCAs tested [four biofungicides: Binab T-vector, Binab TF WP and Binab TF WP (all three *Trichoderma harzianum* ATCC 20476 and *Trichoderma polysporum* ATCC 20475), and Trianum (*Trichoderma harzianum* T-22); and three biological insecticides: Botanigard (*Beauveria bassiana* GHA), Naturalis (*Beauveria bassiana* ATCC 74040) and Preferal (*Paecilomyces fumosoroseus* APOPKA 97)], were

used at their respective MFRC and stored in accordance with the manufacturers' guidelines.

Risk assessment bioassay for toxic and sublethal effects by BCAs: Adult workers were exposed to the different BCAs at their respective recommended MFRCs by topical application, and also orally via drinking treated sugar water or treated pollen. This setup has already proven its usefulness in biomonitoring for side-effects by different classes of chemistry (Mommaerts *et al*, 2006a & b). In brief, artificial nests of workers were treated, each containing five workers. The nest was then followed for a period of about 10 weeks. To evaluate acute bee toxicity, the numbers of dead workers were scored on a weekly basis. In addition, the numbers of drones were counted weekly per nest as a biological endpoint for effects on insect growth, development, brood care and reproduction.

Results

Biofungicides: For the three Binab products when dosed at their MFRC either by topical contact or by oral feeding of treated sugar water or pollen, no toxicity was scored for the worker bees up to the end of the experiment, i.e. 11 weeks. The cumulative numbers of drones in all these tests scored did not differ significantly with the control nests ($P>0.05$). In contrast, in the nests exposed to Trianum at its MFRC by topical contact and via drinking sugar water, the worker mortality was 47% and 31%, respectively. But, when Trianum was treated orally at its MFRC via pollen, it was not toxic and there was no effect on reproduction. Contact with Trianum had strong negative effects on reproduction as the number of drones was only 28% of that in the control nests ($P<0.001$); oral exposure via sugar water and pollen was less harmful with a respective reduction of 56% and 55% compared to the controls ($P<0.01$).

Biological insecticide: Botanigard killed 90% of the workers treated by contact and as a consequence this was detrimental for reproduction (only 9% compared to the control). Oral exposure via sugar water and pollen resulted in mortalities of 29% and 0%, and reproduction of 77% and 90% compared to control values, respectively. For the other two insecticides, the percent mortality of workers was in all cases <20%. The number of drones produced by contact, sugar water and pollen yielded 76, 100 and 71% of that of the control groups (Naturalis) and 95, 59 and 94% (Preferal), respectively.

Conclusions

Overall, the results obtained under laboratory conditions indicated that the three Binab products tested are safe to be used in combination with *B. terrrestris*; the bio-fungicide Trianum can be used, however, only with caution. For the biological insecticides, it was clear that Botanigard was detrimental for workers by contact and sugar water. However, it should be noted that the current tests are worst case and very severe, and it is unlikely that bumblebees will be exposed to such high concentrations in practice. For drawing a final conclusion we suggest that the tests should be performed under more realistic conditions. The other two insecticides tested showed results that allow a combined use with bumblebees.

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The extent of natural bio-control of powdery mildews by hyperparasites

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Stratified random sampling surveys were conducted in the campus of the University of Reading for 2½ years, from October 2004 until October 2006. Aims of the survey were: to investigate the size and the variability of powdery mildew populations on oak trees of different ages, located in two different locations; to explore the incidence of fungi associated with *E. alphitoides*; to identify those fungi, explore their association with *E. alphitoides* and estimate the frequency and severity of mildew infection by each one; to relate the disease severity, the age and the location of the trees to the presence of the fungi associated with *E. alphitoides* and explore the possible interactions and relationships among them; to investigate if the recorded differences in the disease severity levels throughout the different seasons are due to climatic factors or due to the incidence of the fungi associated with *E. alphitoides* or to a combination of these two factors.

Assessments of disease severity were made twice per year, one in mid-summer and one in autumn. On each tree, powdery mildew disease severity was assessed for 50 leaves on a 0-100% scale. Assessments of fungi parasitic on *E. alphitoides* were made by using sellotape strips, prepared from the upper and lower leaf surface of mildew infected leaves (10 leaves collected from two arbitrarily selected branches of each assessed tree). Observed parasitic/antagonistic fungi were rated on a 0-3 scale, where 0=absent and 3=abundant parasite.

Four genera co-existed commonly with *E. alphitoides*. Three out of four symbionts were identified as *Acremonium* sp., *Trichoderma* sp., and *Leptosphaerulina* sp.. The fourth symbiont was identified as *Ampelomyces-Phoma* sp., because the distinction between those two groups is extremely difficult under light microscopy conditions. The sources of variation in abundance of fungi parasitic on *E. alphitoides* were investigated using variance component analysis. The following random factors were considered: the year of specimen collection; the selection of the trees in each location; and the collection of the branches/tree. Fixed factors considered were: location; height-class (age) of the trees; the leaf surface; and the season of specimen collection.

For *Trichoderma* sp. the variance due to differences between leaves within branches was almost equal to the sum of variances due to differences between trees in one location and for the different years. The populations of *Ampelomyces-Phoma* sp. were affected greatly by the differences between years (presumably due to climatic factors) and between trees within locations; this effect was as large as the differences between the leaves within a branch. In contrast, for *Leptosphaerulina* sp. most of the variance observed arose from differences between leaves within branches, so *Leptosphaerulina* sp. severity was mainly affected either by very small-scale environmental differences or chance demographic effects. In addition, *Leptosphaerulina* sp. was the parasite which was the least affected by differences due to year effect or differences between trees within locations. Variance in *Acremonium* sp. severity was affected considerably by the differences between trees within locations and year, but mostly by differences between leaves within branches.

Ampelomyces-Phoma sp. abundance was always greatest in autumn. The populations of *Trichoderma* sp. built up from summer to autumn and they were quite variable in different years and seasons. *Leptosphaerulina* sp. was abundant every year and its populations were higher in autumn. In contrast *Acremonium* sp. was most frequent in summer.

Leptosphaerulina sp. was commonly found both alone or in co-existence with all the other parasitic fungi. *Trichoderma* sp. severity was increased in the presence of *Leptosphaerulina* sp.. The same trend was also seen in the significant three way interaction between *Trichoderma* sp., *Leptosphaerulina* sp. and *Acremonium* sp.; in the absence of *Acremonium* sp. and the presence of *Leptosphaerulina* sp., the populations of *Trichoderma* sp. were increased. Abundance of *Leptosphaerulina* sp. was also greater if *Acremonium* sp. was present. This association was particularly interesting since it seems that there was a synergistic build up effect for the populations of the two parasites. *Trichoderma* sp. shares a unique relationship with *Acremonium* sp.. At the start of this study, they rarely occurred together. However, this relationship was reversed through the years and *Trichoderma* sp. was observed more frequently than expected in the presence of *Acremonium* sp.. This was paralleled by reduced populations of *Acremonium* sp. but increased populations of *Trichoderma* sp.

Leptosphaerulina sp. was the most commonly observed hyperparasite on *E. alphiotides*, but has not previously been recorded as a hyperparasite of powdery mildews. It does not seem to produce conidia and the pseudothecia, which comprise the sexual stage of this parasite, could account for the efficient persistence of this parasite through the years. *Leptosphaerulina* sp. had particularly strong interactions with the other parasites recorded. Of particular interest was its synergistic association with *Acremonium* sp.. Does this association arise because *Acremonium* sp. uses *Leptosphaerulina* sp. pseudothecia as a niche on which to over-winter? If so, it may also be attached to discharging ascospores and therefore be transported to *E. alphiotides*. A similar question applies also to the relationship between *Trichorerma* sp. and *Leptosphaerulina* sp.. Populations of *Trichoderma* sp. were higher in autumn, but *Acremonium* sp. were more common in summer. This could be just due to their different life-cycles or due to associations, relationships and interactions between the symbionts or between each symbiont and its host. Experiments on the interactions are in progress.

In general, it is currently difficult to make definite inferences about the ecology and the life-cycle of each of the studied parasites. The difficulties that have been encountered are mainly due to the complexity of the studied system and the lack of information for all the hyperparasites, which have not been studied in detail.

Enhancing the biological control of leafroller pests in caneberries with proper pesticide timing

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The orange tortrix (*Argyrotaenia franciscana* Fernald) is a leafroller that has been a principle contaminant in machine harvested caneberries since the advent of broad spectrum pesticides in the 1950's. Many fields however experience little or no risk of orange tortrix larval contamination, and this is believed to be due to normally very low endemic populations that are held in check by natural enemies. Our goal for this project is to study and better understand the development and phenology of the leafroller pests and their natural enemies, particularly the parasitic or parasitoid wasps, so that selective and, if required, broad spectrum pesticides can be used without triggering long-term build-up of the leafrollers.

This ongoing four year project aims to: determine the incidence, timing and activity levels of the key parasitoid species in caneberry fields with different pesticide programs in Western Oregon and Washington; investigate the direct effects of pesticides on these species in laboratory bioassays; and to develop improved monitoring practices and phenological models for key leafroller and parasitoid species.

A total of 120 fields in 2005 and 100 fields in 2006 were monitored for leafroller larvae and adults, and 7,291 leafroller larvae were collected. Orange tortrix had 24.4% and 36.6% total parasitism in 2005 and 2006, respectively, and another leafroller which is attacked by some of the same parasitoid species, the oblique-banded leafroller (*Choristoneura rosaceana* Harris), had 19.3% and 20.5% parasitism, respectively. Parasitism was consistently higher in fields that did not have broad-spectrum pesticides applied and the numbers of orange tortrix larvae found in these fields were lower. The braconid wasps *Apanteles aristoteliae* Vier. and *Meteorus argyrotaenia* Johan. were responsible for over two-thirds of the orange tortrix parasitism and over half of the oblique-banded leafroller parasitism.

A culture of *A. aristoteliae* has been established, and laboratory bioassays of the insecticides most commonly used in caneberry fields were conducted in arenas with dried deposits of various rates of these materials (see Table 1).

Due to the fact that these bioassays may not accurately represent the amount of insecticide exposure experienced in the field, bioassays are also being conducted in field inclusion cages in commercial fields. In these trials, the effects of residues of various spray treatments on *A. aristoteliae* wasp mortality and oviposition activity, as well as on the mortality of *A. franciscana* larvae are being assessed. The results of these trials will be used in conjunction with phenological models of parasitoid activity (see below) to provide growers with recommendations about how to avoid treating during times of peak parasitoid activity.

Table 1. Mortalities of *A. aristoteliae* wasps exposed to different insecticide treatments

Treatment		% Mortality*			
Active ingredient	Rate / acre	1 hour	6 hours	24 hours	48 hours
Bifenthrin	0.64 oz (1/10 field)	86.67	100.00	100.00	100.00
Bifenthrin	0.064 oz	76.67	80.00	100.00	100.00
Bifenthrin	0.0064 oz	23.33	70.00	80.00	85.19
Bifenthrin	0.00064 oz	0.00	6.67	27.59	34.62
Bifenthrin	0.000064 oz	0.00	0.00	0.00	15.38
Malathion	0.64 oz 1/10 field)	100.00	100.00	100.00	100.00
Malathion	0.064 oz	66.67	93.33	100.00	100.00
Malathion	0.0064 oz	0.00	70.00	93.33	100.00
Malathion	0.00064 oz	0.00	3.45	6.90	1.89
Pyrethrum	6.4 oz (1/10 field)	75.86	--	100.00	100.00
Pyrethrum	0.64 oz	6.67	--	10.34	10.34
Pyrethrum	0.064 oz	0.00	--	4.21	8.05
Spinosad	6.0 oz (field rate)	0.00	90.00	100.00	100.00
Spinosad	0.6 oz	0.00	20.00	100.00	100.00
Spinosad	0.06 oz	0.00	20.00	--	3.31
Tebufenozide	160.0 oz (10x field rate)	0.00	0.00	--	--
Tebufenozide	16.0 oz	0.00	3.33	--	--
Tebufenozide	1.6 oz	0.00	0.00	0.00	0.00

*Corrected for control mortality using Abbott's formula.

We are moving from a general understanding of leafroller and parasitoid phenology to a more quantitative framework, with phenological models. Temperature development studies of the key parasitoid species are being conducted so that the time of attack of field-collected specimens can be back-calculated, and their time of emergence can be forward-calculated. The peak periods of leafroller and parastoid abundance in the field can then be used to assess the periods of greatest pesticide susceptibility.

This information will then be used to produce a calendar of recommended scouting activities and updated guidelines for leafroller management in caneberries. Other project activities include several outreach events, improving leafroller and parasitoid monitoring techniques, studies of novel post harvest leafroller contaminant removal methods, and formal evaluations of the risks and economics of new IPM methods versus conventional practices. Together these technologies may provide the tools to reduce leafroller populations down to low endemic levels by promoting IPM and conservation biological control in caneberries.

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Resistance in crops: metabolism and transport of xenobiotics in the rhizosphere

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Introduction

Agricultural plants are exposed to foreign compounds via the atmosphere, in aqueous solution or as particles by chance, or in the context of agricultural activities. Resistance to herbicides has evolved in numerous species by induction of effective detoxification mechanisms. These mechanisms include production of potent scavenger molecules, but also the expression of defence enzymes, like the P450, glucosyl transferase and glutathione transferase super-families. By analogy to mammals, the resulting detoxification network has been named green liver. Herbicide resistance in plants is to a large extent based on the availability of these metabolites and the presence of the respective enzymes. It is generally accepted that plants build up soluble or bound residues after detoxification, i.e. from phase II products. Main storage pools are thought to be the vacuoles and the cell walls of plant tissue. However, glutathione dependent detoxification leads to the formation of conjugates that are cleaved to form numerous soluble products. These reactions are partially catalyzed in the cytosol, but also in vacuoles. It is of great interest for risk assessments to know whether the resulting metabolites remain soluble and whether they might exert some effects on the plant or other organisms.

Materials and methods

Synthesis of conjugates – Glutathione-derived metabolites were synthesised by mixing GSH or cysteine in 100 mM Tris/HCl buffer, pH 7.8 with CDNB at room temperature for two hours. Resulting GS-DNB (S-(2,4-dinitrophenyl)-GSH) and Cys-DNB (S-(2,4-dinitrophenyl)-CYS) were verified by HPLC and purified *via* TLC (BuOH: HAc: H_2O, 12:3:5).

Barley seedlings – Seeds of *Hordeum vulgare* (var. Cherie) were surface sterilized and germinated in the dark at 20°C in Petri dishes on filter paper.

Fungal cultures – *Chaetomium globosum* and *Trichoderma ssp.* isolated form agricultural soils were precultured for four weeks in malt extract. For incubations with xenobiotic conjugates, mycelia were transferred to malt extract agar preinoculated with GS-DNB and Cys-DNB, respectively, for 24 hrs.

Enzyme assays – Glutathione S transferase assays were performed according to published methods (Schröder *et al.*, 2007).

Transport studies – Transport of GS-X and derived conjugates through barley roots was measured in Pitman-chambers, as published recently (Schröder *et al.*, 2007).

Results and discussion

Sequestration of xenobiotics and their metabolites in plants has been the subject of several studies (Wolf *et al.*, 1996; Schröder, 2001), and glutathione transport and cell-to-cell transport of xenobiotic conjugates has been demonstrated. We report about unidirectional long range transport of xenobiotic conjugates through plant tissues. It can be assumed that conjugates will, after their formation in aerial tissues, diffuse into the apoplast and be transported into the root. Simultaneous transport of GS-DNB and Cys-DNB was investigated to explore the potential for exudation of these conjugates. An expected ratio

for diffusive transport of GS-DNB over Cys-DNB would then be 0.78, taking into account that the larger glutathione conjugate would move slower inside the vessels and through potential exudation pores. However, the relative transport velocity of the GS-conjugate above the Cys-conjugate is significant (Table 1). This observation indicates that a preferred transport of the GS-conjugate over the Cys-conjugate takes place, which points at a possible signal function of GS-conjugates (Schröder & Stampfl, 1999, Schröder *et al.*, 2007).

Table 1. Transport velocities of GS-DNB and CYS-DNB in plant and root tissue

Conjugate	tissue	Rate/min	fold incr.
GS-DNB	whole plants	0.0503	
Cys-DNB	whole plants	0.0190	2.6
GS-DNB	roots with tips	0.0565	
Cys-DNB	roots with tips	0.0232	2.4
GS-DNB	roots tipless	0.0486	
Cys-DNB	roots tipless	0.0449	1.1

In the rhizosphere, exuded conjugates alter the behaviour of soil fungi. Both, *Chaetomium* and *Trichoderma* showed strong reactions in their GST activities over time when grown on agar inoculated with GS-DNB or CYS-DNB (Table 2). Interestingly, their reactions are different from each other, which indicate differences in the signalling pathway.

Table 2. GST activity in soil fungi grown on agar spiked with GSH derived conjugates: change in GST activity [% of controls at t=0]

[hr]	3	6	9	3	6	9
Substrate		+ GS-DNB	*Chaetomium*		+ CYS-DNB	
CDNB	200	100	308	180	180	150
NBC	96	110	240	0	100	150
NBOC	20	48	72	20	23	116
			Trichoderma			
CDNB	79	106	65	280	300	100
NBC	91	84	38	180	170	15
NBOC	78	69	16	122	200	144

Studies are underway to elucidate further possible impacts of xenobiotic metabolites on rhizosphere bacteria and their interactions with plant roots.

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Purification and comparison of anti-feeding proteins between extracellular and intracellular protein of *Xenorhabdus nematophila* var. *Pekingensis*

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Introduction

Xenorhabdus nematophlia var. *pekingensis* CB6 strain is a symbiotic bacterium of *Steinernema carpocaposae* isolated from the soil of Beijing in China. This bacterium lives in the gut of the host nematodes. It is released from the gut of its host nematode to the insect hemocoel when the nematode penetrates into insect larvae. The bacteria multiply and secrete various kinds of metabolic substances, killing the insect and inhibiting the growth of other microorganisms. The CB6 strain showed high growth inhibition and high mortality with the larvae of *Helicoverpa armigera*, *Plutella xylostella*, *Spodoptera exigua*, et al. (Liu *et al.* 2003Li *et al.* 2003). The bioactive substances were found in both extra-cellular and intracellular sections. Heat treatment or enzymatic degradation inactivated the bioactivity. This indicates that the active substances are proteins (Pan *et al.* 2004).

In this paper, the purification and comparison of anti-feeding proteins from extra-cellular and intracellular sections are reported. It provides fundamental data for further research on anti-feeding proteins, the cloning of this insecticidal protein and its mode of action against insects.

Methods

X. nematophila var. *pekingensis* strain CB6 was cultured in a 100 mL shaking flask at 28°C for 48 h. The cell broth was centrifuged at 12,000r/m for 10 min. The supernatant was collected (extra-cellular substance A1), and the remaining bacterial cells were then suspended in the same buffer with sterile PBS and these steps repeated three times. The cells were pulverized with an ultrasonic pulverizer (65 amplitude, 6s running and 6s pause for 11 min), centrifuged at 12,000 r for 10 min and the supernatant collected (intracellular substance A2). The extracellular and intracellular fractions A1 and A2 were collected and precipitated with ammonium sulphate. The optimal saturation to precipitate the active proteins was determined on the basis of an insect bioassay.

DEAE-Sepharose FF chromatography, Butyl Sepharose FF chromatography, and Sephacryl S-200 HR chromatography were used for purification. The protein components were analyzed using the techniques of native-polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Periodic acid–Schiff (PAS) staining was used for identification of glycoprotein. The native-PAGE gels were stained for the detection of lipoprotein.

Results

The optimal range of ammonium sulphate for precipitating the toxic proteins was 35% to 50% saturation. The fractions A1 and A2 precipitated under 30 to 50% saturation were re-suspended in PBS buffer, filtrated and salted out by a Hiprep 26/10 Desalting column equilibrated with 100 mL 25 mM Tris-Hcl buffer (Buffer A), pH 7.4. The eluted protein peaks were fractions B1 and B2. The 5mL fractions of B1 and B2 were concentrated and applied to a DEAE column, which was equilibrated with 25 mM Tris-Hcl buffer (pH 7.4).

Samples of each of the eluted protein peaks were tested for bioactivity. Activity was found only in the peaks C1 and C2. C1and C2 were collected from the elution and salted out by a gel filtration HiPrep Desalting column and applied to a butyl FF sepharose column, and eluted in a linear gradient from 0 to 3 M NaCl. The peaks D1 and D2 were the active fractions based on bioactivity assays. Bound protein fractions D1 and D2 were further concentrated and applied to a gel filtration sephacryl S-200 HR. Activity was found only in the small peak eluted from the column at approximately 112~115 min, (active peak fractions E1 and E2). Native-PAGE for purified fractions E1 and E2 gave the same band, with a molecular weight greater than 669 kD. The bands of SDS-PAGE for E1 and E2 were seen as a single band in Coomassie brilliant blue stained gel with the molecular weight greater than 212kD. The results indicated that the purified proteins E1 and E2 might be homogeneous. The purification steps are summarized in Table 1.

The growth inhibition of E1 and E2 against *Helicoverpa armigera* Hübner larvae was 62.63±19.2185and 97.90±0.1569% at concentrations of 2.58 μg mL^{-1} and 4.21 μg mL^{-1}, respectively. The growth inhibition of E1 and E2 per μg protein was 37.28±11.4396% and 23.25±0.0373%, respectively, which showed that the toxicity of E1 was similar to that of E2. E1 and E2 still maintained inhibitory toxicity after being heated up to 60. However, if they were heated up to 80 or 100, the inhibitory toxicity was lost. Staining experiments showed that the toxic protein was neither lipoprotein nor glycoprotein.

Table 1. A summary of toxicity protein purification for E1

Purification steps	Concentration (μg mL^{-1})	growth inhibition (□)	growth inhibition per μg protein (□)
Extracellular crude protein	469.66	88.65±2.6903	0.19±0.0057
Ammonium sulfate Precipitation)	84.70	95.32±0.9977	1.13±0.0118
DEAE-Sepharose	18.71	92.83±1.3900	4.96±0.0743
Butyl sepharose fast flow	4.82	97.74±0.1255	20.28±0.0260
Sephacryl S-200 HR	2.58	62.63±19.2185	37.28±11.4396

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An *In vitro* study on biological potantion of some Iranian Trichoderma isolates in control of soil borne plant pathogenic fungi

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In the recent years, there has been a world wide swing to the use of eco-friendly methods for protecting the crops from pest and disease. Antagonistic nature of fungal species from the genus Trichoderma against some economically important aerial (Elad, 2000) and soil borne plant pathogens (Clavet *et al.*, 1990, Papaviza, 1985) are well documented. Possible mechanisms of antagonism by *Trichoderma spp* including nutrient and niche competitions, antibiosis (producing volatile components and non volatile antibiotics) that were active inhibitory to against a range of soil borne fungi, as well as parasitism (Dennis and Webster, 1971a, b, c). Selection of biocontrol agents as well as understanding the mechanisms involved in the antagonistic effect of *Trichoderma spp* on plant pathogens are important in designing effective and safe biocontrol strategies.

In this study the *in vitro* potential of six selected Iranian isolates of three species of Trichoderma (*T. hamatum* T614, *T. hamatum* T612, *T. harizianum* T447, *T. harizianum* T969, *T. virens* T523 and *Trichoderma sp*) were evaluated against five isolates of soil borne phytopathogenic fungi (*Fusarium graminearum*, *Rhizoctonia solani* (AG4 & AG5), *Macrophomina phaseoli* and *Phytophtora cacturum*) in dual culture techniques and through production of volatile and non-volatile inhibitors Dennis & Webster (1971b,c), respectively. Temperature and pH effects on Trichoderma isolate growth were also studied.

All Trichoderma isolates had a marked statistical inhibitory effect on mycelial growth of the pathogens in dual culture compared with controls. Maximum inhibition was occurred in *F. graminearum-T.hamatum* T614 interactions in compared with tested interaction. In dual cultures of *T. virens* T523 and *T. harizianum* T969 there was an inhibition zone without physical contact between the colonies around all pathogen colonies. An inhibition zone without hyphae contact was observed in *R. solani* AG4 & AG5-*T. harizianum* T447 and *P. cactorum-Trichoderma sp* interactions. No inhibition zones with other pathogen-Trichoderma interactions were observed.

Significant pathogen colony growth inhibitions were observed when exposed to the trapped atmosphere from culture of the Trichoderma. *F. graminearum* was most susceptible to the volatile inhibitors produced by *T. hamatum* T612 (% inhibition = 48.65). Minimum inhibition occurred with the *M. phaseoli- T. hamatum* T614 interaction (% inhibition = 10.86).

Medium filtrate obtained the Trichoderma isolate culture also were effected on the pathogen species significantly. Maximum growth inhibition were observed in radial growth of *Fusarium graminearum* by *T. hamatum* T612 non volatile metabolites (% inhibition =

38.3). Among *M. phaseoli* and *R. solani* AG4 were recorded minimum growth inhibition by non volatile inhibitors of *T. hamatum* T614 and *Trichoderma* sp T respectively (% inhibition = 6.5).

In addition, this study revealed that the Trichoderma isolates have an effective performance in control of the pathogen colony growth. Different isolates of Trichoderma have various strategies for fungal antagonism. *T. hamatum* T612 are good effect for controlling *F. graminearum* in each tested strategies that employed by Trichoderma isolates and can a suitable candidate for our further study in field conditions against *F. graminearum*.

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