Session 6D

Efficacy of Biological Control, Using Living Organisms and Natural Products

Multitrophic Interactions

Chairman:	Dr Eunice Allan *University of Aberdeen, UK*
Session Organisers:	Dr Eunice Allan *University of Aberdeen, UK* and Dr Annegret Schmitt *BBA, Darmstadt, Germany*
Platform Papers:	6D-1 to 6D-4
Poster Presentations:	P6D-5 to P6D-36

Complex multitrophic interactions in the plant environment can affect disease biocontrol

J M Whipps
Warwick HRI, University of Warwick, Wellesbourne, Warwick, CV35 9EF, UK
Email: john.whipps@warwick.ac.uk

With increasing pressure to reduce chemical use in the environment, and legislation decreasing the number of active molecules available for use, there has been considerable interest in developing alternative non-chemical methods of disease control. Such biological procedures for control of plant pathogens have generally been targeted at the identification, development and commercialization of specific microbial inoculants as substitutes for chemical fungicides. This approach has resulted in some success with several viruses, over 30 bacteria and more than 50 fungal products on sale worldwide for control of a number of soilborne, foliar and post-harvest diseases (http://www.agr.gc.ca/env/pdf/cat_e.pdf; http://www.oardc.ohio-state.edu/apsbcc/; Whipps & Gerhardson, 2007). Nevertheless, this number of commercial products is relatively small in comparison with chemical fungicides. This reflects a combination of factors: relative level of reproducibility and efficacy; problems with scale-up; shelf life and quality control; cost; and registration and clearance for commercial use, particularly for what are often niche markets. Of these, the perceived poor level of reproducibility and efficacy is the major problem and this is due to a failure to understand and appreciate the modes of action and complex ecology of the biocontrol agents (BCAs) and the interactions that take place with the host and target pathogen(s). All these are further complicated by the influence of environmental and microbiological factors which can differ at the level of the plant as well as the site of cultivation.

Numerous modes of action of BCAs have been identified over the years. Those that work directly on the pathogen include: competition for nutrients, space or infection sites; antibiosis; parasitism; production of cell wall-degrading enzymes; and degradation of pathogenicity factors. These can occur associated with the plant or distant to the plant. Others work indirectly through specific effects on the plant only and include induced resistance and plant growth promotion. The complexity of this situation is further compounded when it is realized that in any one BCA-pathogen-plant combination multiple modes of actions may occur simultaneously or with time – none are necessarily mutually exclusive.

The ecological behaviour of the BCA can also be complex. Some BCAs are rhizosphere competent and can grow or spread down the root from a simple seed treatment providing long-term control from root infecting pathogens whereas others simply survive on the seed and provide short-term control from damping-off pathogens. Other BCAs may have different ecological attributes which affect the host range. For example, the fungus, *Coniothyrium minitans*, is a BCA used against sclerotia (fungal resting bodies) of the widespread plant pathogen *Sclerotinia sclerotiorum*. In nature, this BCA can survive in soil for several years between hosts but has no ability to grow saprotrophically. In contrast, isolates of the fungus *Trichoderma* can also attack sclerotia of *S. sclerotiorum* but they are also capable of growing saprotrophically in soil in the absence of they host. *Trichoderma* spp. may also have a broad host range, able to attack a number of plant pathogens, making them attractive for wider development as BCAs. The only downside to this is whether mass introduction of *Trichoderma* will have any long-term adverse effects on the natural

microbiota of the soil. Clearly, understanding the ecological behavior of BCAs can influence the type and range of pathogens that may be targeted.

Another feature complex interaction that needs to be considered is that between the pathogen and the plant. For example, *S. sclerotiorum* survives between crops as sclerotia and then given the correct environmental and nutritional stimuli, the sclerotia may germinate either to produce mycelia, which go on to infect the plant directly, or to produce apothecia (fruiting structures) which release ascospores, and these go on to infect the plant. The latter requires the presence of a food source such as plant nutrients or senescent material for infection to take place So where to apply the BCA? Against sclerotia or ascospores? To utilize the food base and make it unavailable to the pathogen? To prevent sclerotia production on senescent infected tissue? Certainly there is no easy single target and all these approaches have been examined. This can be even more complicated when the environmental factors are considered. Apothecia are only produced within a temperature range between 5-25°C and when there is sufficient water available. Dry Spring weather during flowering frequently results in little Sclerotinia disease on oilseed rape as few apothecia are formed and infection depends on petal infection by ascospores. However, the sclerotia remain in the soil and can germinate the following year if the environmental conditions are appropriate.

Animals can also influence the interactions that can take place. For example, mites and collembolans have been shown to transfer *C. minitans* from infected sclerotia of *S. sclerotiorum* to uninfected sclerotia in soil, thereby potentially enhancing the efficacy of the BCA. Soil fauna have also been reported to consume sclerotia and provide some direct control of *S. sclerotiorum*. Interestingly, bees are now routinely used to carry *Trichoderma* and *Gliocladium* species to flowers and foliage of plants for the control of *Botrytis cinerea* showing how complex multitrophic interactions can be utilized.

So far, all the emphasis has been concerned with disease control associated with the use of microbial inoculants as BCAs. However, the possibility exists to utilize cultural and environmental procedures to provide disease control and many of these involve highly complex effects on multitrophic interactions. These include: plant resistance; crop rotations; soil amendments, such as incorporation of organic matter and composts; and development of suppressive soils and recirculating growing systems. Although many of these procedures are rather 'old-fashioned' they may be the only methods available for some organic growers. It is also possible to integrate these approaches with BCAs to provide long-term and more sustainable disease control which may be the way ahead.

Reference

Whipps JM; Gerhardson B (2007) Biological pesticides for control of seed- and soil-borne plant pathogens. In: *Modern Soil Microbiology 2nd Edition* (Eds. JD van Elsas; J Jansson; JT Trevors), pp. 479-501, CRC Press LLC, Boca Raton.

The *Ephelis* fungus, an epiphytic symbiont of C4-grasses, confers resistance against herbivorous pests and environmental stress

T Tsukiboshi, R Uegaki, K Sugawara
National Institute of Livestock and Grassland Science, Nasushiobara, Tochigi, Japan
Email: tuki@affrc.go.jp

S Yoshimatsu
National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan

Black choke is a well-known disease of gramineous plants including rice causing sterile heads with black to grey stromata of mummified appearance. The disease is common especially in India and China and called 'Udbatta' and 'I-Chu-Hsiang' disease of rice, respectively. Many C4 warm season grasses have also been reported as hosts, such as *Echinochloa crus-galli* (barnyard grass), *Setaria italica* (foxtail millet), *Pennisetum americanum* (pearl millet), *Sorghum vulgare* (sorghum) and others, mainly in India, China and in the Americas. In Japan, the disease has been found on many species of C4-grasses, mainly in the southern region. In addition to the typical black choke symptom, most of the infected grasses have a sign of white streaks of hyphae on the leaves showing the epiphytic characteristics of the pathogenic fungus. The purpose of the paper is to characterize the causal fungus and study its epiphytic relationship with the infected plants in addition to the beneficial effect to the infected plants such as insect resistance and cold tolerance.

Table 1. Hosts, origins and characteristics of the *Ephelis* fungus causing black choke and/or leaf streak symptoms in Japan

Isolate no.[a]	Host grass	Geographical origin in Japan	Isolated from	RFLP Type[b]	Conidial length (μm)	Optimum temp. °C
306585	*Chloris barbata*	south	head	A	9.8-27.9	25
306575	*Chrysopogon aciculatus*	south	head	B	12.3-23.5	25
306578	*Cynodon dactylon*	south	head, leaf	B	13.2-25.0	28
306582	*Cynodon pletostachyrus*	south	leaf	A	7.8-21.6	20
306587	*Digitaria eriantha*	south	leaf	A	12.3-20.6	25
306581	*Digitaria violascens*	south	head, leaf	A	8.8-24.5	25
306577	*Eragrostis ferruginea*	central	head, leaf	A	16.2-23.5	28
306625	*Eriochloa procera*	south	head	B	11.3-26.1	-
306583	*Imperata cylindrica*	central	head, leaf	A	14.7-25.5	25
306624	*Panicum repens*	south	head	B	14.8-24.6	-
306576	*Paspalum orbiculare*	south	head, leaf	A	14.7-19.1	25
306584	*Paspalum thunbergii*	central	head, leaf	A	11.8-21.6	28
306580	*Pennisetum alopecuroides*	central	head	A	16.2-19.6	28

a: The isolates were deposited at the genebank of the National Institute of Agrobiological Sciences (NIAS), Japan.

b: Type A produced bands of about 370 and 200 bp, and type B of about 220, 200 and 150 bp by RFLP analysis of rDNA-ITS regions digested by *Mbo*I.

Samples of C4-grasses of 19 species belonging to 14 genera in four families of grasses showing black choke and/or leaf streak were collected from Ishigaki Island, the most southern region and from Tochigi Prefecture, the central region of Japan. On the stromata of diseased heads and leaves, palisades of conidiophores terminating in narrow phialides produce many conidia on the tips. The conidia are colorless, needle-shaped, non-septate and 7.8-27.9 x 0.9-2.7 µm. As shown in Table 1, there was no relationship between conidial length and geographical origin or host of the fungal isolates. The optimum temperature for mycelial growth varied mostly between 25 and 28 °C but there was also no relationship between optimum temperature and geographical origin. No teleomorphs of the fungus were observed in nature. Based on conidial morphology, all isolates from the collected plants were distinctly assigned to the genus *Ephelis*, the anamorph of *Balansia* (Clavicipitaceae). The RFLP patterns of rDNA-ITS regions were analyzed by digesting the PCR products with the restriction enzyme *Mbo*I. The results revealed the presence of type A of *Ephelis* causing black choke including *B. andropogonis* and type B including *B. discoidea* and *E. oryzae* as we reported before (Tanaka *et al.*, 2001). Type A was found only on isolates from the southern area, whereas type B was also found on those from the central area of Japan. This may indicate the actual distribution of the two groups of *Ephelis* in Japan.

In *Ephelis*-infected knotgrass (*Paspalum thunbergii*), hyphae of the fungus colonized the surface and basal part of leaf primordia and surrounding tissues. However, the leaf primordia appeared intact without visual damage by the hyphae. By scanning electron microscopy (SEM), it was observed that many strands of hyphae with conidial masses extended epiphytically on the surface of the leaves of *Ephelis*-infected plants. These characteristics show that the fungus should be an epbiont as reported previously for *B. andropogonis* and *B. dicoidea.*

Ephelis-infected plants of knotgrass were treated with the systemic fungicide Triforine. Surviving plants were checked for the presence of the fungus, and the symptomless ones were kept as epiphyte-free clones for the following experiments. In order to evaluate the effect of the epiphyte on the feeding behaviour of insects, leaves of infected and non-infected plants were fed to rice grasshoppers (*Oxya yezoensis*) and sedge tussock moths (*Laelia coenosa sangaica*). It was observed that the presence of *Ephelis* on leaves deterred the two herbivorous pests from feeding. For estimation of their cold tolerance, the same set of plants was cultivated in the field from June to December in 2002. Survival of the *Ephelis*-infected plants was enhanced compared to *Ephelis*-free ones under the cold conditions in the field.

The results of our study indicate that the *Ephelis* fungus is potentially suited as a symbiont of C4-grasses for biological control of diseases, pests and environmental stress, similar to *Neotyphodium* as endophyte of C3-grasses. It should be possible to utilize the fungus for controlling pests or diseases in the original hosts, bermudagrass (used as turf grass), paragrass and giant stargrass (both used as forage crops). However, an inoculation method of the fungus has never been developed. Therefore, further studies are necessary for the utilization of the *Ephelis* fungus as a symbiont.

Reference

Tanaka E, Kawasaki S; Matsumura K; Kusuda R; Tanaka C; Peng YouLiang; Tsukiboshi T; Tsuda M (2001): Phylogenetic studies of *Ephelis* species from various locations and hosts in Asia. Mycological Research **105**, 811-817

An immunological axis of biological control: microbial infections in field-caught insects

D W Stanley
USDA Agricultural Research Service, Biological Control of Insects Research Laboratory, 1503 S. Providence Road, Columbia, MO 65203, USA
Email: stanleyd@missouri.edu

H Tunaz
Kahramanmaraş Sütcü Imam University, Kahramanmaraş, Turkey

Many species of naturally occurring entomopathogenic microbes are virulent insect pathogens, including viruses, fungi and bacteria. Some of these disease organisms serve as important natural regulators of insect populations by invading insect bodies, often by way of the alimentary canal. Appreciation of insect diseases and the possibilities of using insect disease agents in biological control programs have a long history. Commercially useful microbial pathogens include viruses, fungi, bacteria, protozoans and nematodes, all deployed as agents in biological control of insect pests, weeds and plant diseases.

The relative success and failures of microbial control of insect pests ultimately depends on a wide range of factors, including costs, the context of comprehensive IPM programs, education of users, government activities, as well as political and environmental concerns. But from a technical perspective, successful microbial control will depend on biological issues. These issues span a range of biological organization from the ecological level of microbe-host population dynamics to the molecular and cell biology of host defense mechanisms.

One of the important barriers to successful deployments of microbial control agents may lie in insects' robust and complex innate immune effectors. Insect innate immunity is comprised of a number of host defense systems. Once they permeate the physical barriers surrounding insect bodies, invading microbes are confronted with fast-acting cellular defense actions, including phagocytosis and nodule formation. These cellular defense reactions begin immediately after an infection is detected within an insect. Some hours after an infection is detected, insects unleash a daunting array of anti-microbial peptides that constitute the humoral immune system. The combined arsenal of immune effector mechanisms allows insects to either stifle infections at their onset or to overcome invasions and infections. In the context of this paper, these mechanisms also can limit the effectiveness of microbes deployed for biocontrol of insect pest populations.

It is difficult to appreciate the extent to which insect immunity functions to protect insects from microbial infection in nature, where they must arrest small infections or recover from larger ones. Because the potential for infection is high, we formed the hypothesis that most of the insects living in agrarian fields experience natural infections. Here we report on the outcome of a field investigation designed to test our hypothesis.

Insects were collected from fields surrounding the city of Kahramanmaraş, Turkey in 2003-2005, using either hand collection or routine sweep net procedures. The collected species, collection sites, site altitudes, and biological stages are indicated in Results. The specimens were transferred to the laboratory (20 ± 1 °C, RH 60 ±5), where nodulation was assessed.

Nodulation was assessed in a total of 19 insect species collected during spring and summer of 2003, 2004 and 2005. In the broadest description, we recorded nodules in 98% of the 435 specimens examined, although there was a very wide range of nodules/specimen (from 1 nodule/insect to >100 nodules/insect).

Our data on nodules in field-collected insects strongly support our hypothesis that most insects in agrarian fields experience naturally-occurring microbial infections. The low numbers of nodules recorded in many specimens indicate insects may arrest small numbers of invaders before larger infections develop. We recorded larger numbers of nodules in other specimens, from which we infer at least some insects recover from large infections and continue their live cycles. We recorded nodules in virtually all examined insect specimens and more nodules from insects found in soil, a site of significant microbial challenge, than other sites. Our collections included representative of four major orders, from which it appears the findings may apply to most insect species. Insects generally are exposed to microbial challenge throughout their lives and in a great number of cases they somehow check the infections.

The nodulation process is the predominant insect cellular defense action. The final step in nodulation is a melanization action driven by a cellular phenoloxidase. The darkened, melantoic nodules are finally attached to an internal organ or body wall, where they remain through the life of the insect. Because they are not cleared from insect hemocoels, nodules can be taken as an historical record on whether or not any particular insect has experienced a microbial infection. While the absence of nodules would not be proof that an insect is immunologically naïve, the presence of nodules indicates a past infection. For a given bacterial species/insect species set, nodulation is an effective quantitative assessment of the intensity of an infection. For example, in the *S. marcescens*/*M. sexta* system, numbers of nodules was related to the size of bacterial infection in an exponential way ($Y = 0.4955 + 18.33\ X^{0.1558}$). Nodulation has been recorded following infections with bacteria, fungal spores and some viral infections. Infections with some bacterial species evoked far more nodules than similar infections with other species. The presence of melantoic nodules within an insect body is a reliable indicator of past infection, although the numbers of nodules carry no more than hints of information on the size or nature of infections.

The specimens collected for this study appeared to be in good condition in the field. They were moving, consuming food and on inspection their alimentary canals were filled. Specifically, the individuals we examined exhibited the behavior and physical appearance of healthy animals. Apparently, the insects had experienced microbial infections and by the time of our collections had either checked the invasion or had recovered from the infections.

The ability to recover from infections in nature has profound biological and agricultural implications. Biologically, some microbes have evolved mechanisms to evade insect immune surveillance systems. For example, the bacterium, *Xenorhabdus nematophila*, secretes factors that inhibit the eicosanoid signaling crucial to launching cellular immune reactions and secretes an antibiotic responsible for inhibiting phenoloxidase. It would appear that insect immune systems exert selection forces on infecting microbes of sufficient power to influence evolution of mechanisms to avoid insect immunity. A major advance in microbial control technology will be our ability to understand and somehow disable insect immune reactions.

Effect of biocontrol activity on different plant species by *Pseudomonas oryzihabitans* and *Xenorhabdus nematophila* against *Pythium* damping–off

A V Kapsalis, F T Gravanis
Technological Education Institution of Larissa, GR-411 10 Larissa, Greece
Email: Kapsalis_tegos@yahoo.com

S R Gowen
The University of Reading School of Agriculture Policy and Development PO Box 236, Reading, RG6 6AT, UK

Entomopathogenic bacteria are vectored by steinernematid and heterorhabtitid nematodes into insects, where they produce broad-spectrum antibiotics. *Pseudomonas* (=*Flavimonas*) *oryzihabitans* and *Xenorhabdus nematophila* have been reported to offer a promising approach for managing seedling damping-off diseases. Different experimental approaches showed that entomopathogenic bacterial strains (*P. oryzihabitans* and *X. nematophila*) are capable of controlling *Pythium* and *Fusarium* mycelial growth *in vitro* and *in planta* (Kapsalis *et al.* 2003; Vagelas *et al.* 2004). *In vitro* assays indicated production of substances which inhibit growth of the fungi by both biocontrol agents. The aim of this research was to investigate whether biological soil treatment could effectively control cotton, tomato and radish seedling diseases.

In vitro inhibition of *P. ultimum* and *P. aphanidermatum* by *P. oryzihabitans* was measured on PDA plates in a dual culture, as described by Anith & Manomohandas (2001). A 4 mm mycelial plug from an actively growing pathogen culture on PDA was taken and placed at the centre of a 9 cm PDA plate. Four spots of *P. oryzihabitans* and *X. nematophila* suspensions at 10^3,10^4,10^5 and 10^6 cells per ml, respectively, were placed on the edges of the PDA plate 48 h after inoculation by the fungi involved. Control plates with bacteria and fungi alone were inoculated as comparisons. Assessments were made after 4 d by counting a) presence and b) the size of inhibition zones. To investigate *in planta* antagonism (biocontrol), seeds of the tested plants (cotton, tomato, and radish) were dipped in bacterial cell suspension of *P. oryzihabitans* and *X. nematophila,* at different concentrations (10^3, 10^4, 10^5 & 10^6 cells/ml), for 10 mins. The treated seeds were then sown in pots with peat that was inoculated with *Pythium* spp. (10^9 oospores/g) and incubated for 4 weeks (at 26 ^{0}C, 12 h light/dark photoperiod). There were 10 replicates per treatment, with 10 untreated plants as controls.

The bacterial biocontrol concentrations clearly ($P \leq 0.05$) showed a suppressive effect by producing strong inhibition zones against the *Pythium* spp. in the *in vitro* antagonism assay. *X. nematophila* and *P. oryzihabitans* at all four concentrations showed antifungal activity on *Pythium* spp. Plates but especially at the 10^6cells/ml concentration showing significantly better antifungal activity and stronger inhibition zones.

Both young cotton and tomato plants had better growth when treated when treated with *P. oryzihabitans* cells at higher concentrations (10^5 & 10^6 cells/ml). The treated plants were significantly higher in both fresh weight and height, compared with the other treatments and the untreated control for both cotton cultivars (Coker and Aria) as well as the two tomato cultivars (Viomichaniki and Karampola).

Especially in *P. oryzihabitans* (10^6 cells/ml) treatment significant growth on cotton root development in *P. ultimum* infected soil. In radish the 10^6 cells/ml bacterial cell concentration resulted in phytotoxicity from the third week of the incubation period, onwards. Best protection was resulted with 10^4 & 10^5 bacterial cells/ml.

Hendrix & Campbell (1983) stated that soil temperatures affect development of pre- and post- emergence damping-off caused by a number of soilborne pathogens like *Pythium* spp. and *R. solani*. *P. aphanidermatum* was quite pathogenic at 20^{0}C but was generally more destructive at higher temperatures (Tedla & Stangellini, 1992). In the present study the temperature (26^{0}C) effect suggests that seed treatment with *P. oryzihabitans* and *X. nematophila* is a promising method for controlling seedling damping-off in both *P. ultimum* and *P. aphanidermatum*. The treated plants were significantly higher in fresh weight and height, compared with the other treatments and the untreated control in both employed cotton and tomato cultivars, respectively. Radish plants were also protected from Pythium damping-off when the seeds were treated with bacteria (10^4 & 10^5 cells/ml), and were exhibited better growth compared with the other treatments including the untreated control. The application of *P. oryzihabitans* to cotton seeds led to effective control of damping-off caused by *Pythium* spp. under controlled environmental conditions. Especially in *P. ultimum* infected soil which proved to be more pathogenic in lower temperatures than *P. aphanidermatum* the bacterium (*P. oryzihabitans*) concentration 10^6 cells/ml significantly increased root weight compared with the other concentrations. *P. oryzihabitans* treatments efficiently control *Pythium* damping-off symptoms.

Acknowledgments
The first author acknowledges IKY (Greek State Scholarships Foundation) for its sponsorship for his MSc and PhD. All authors acknowledge the involvement of the personnel of University of Reading (Department of Agriculture and School of Plant Sciences) that provided isolates and their help in this study

References

Anith KN; Manomohandas TP (2001). Combined application of *Trichoderma harzianum* and *Alcaligenes* sp. strain AMB 8 for controlling nursery rot disease of black pepper. *Indian Phytopathology* **54**: 335–339.

Hendrix J; Campbell WA (1983). *Pythiums* as plant pathogens. *Annual Review of Phytopathology* **11**: 77-98.

Tedla T; Stanghellini ME (1992). Bacterial population dynamics and interactions with *Pythium aphanidermatum* in intact rhizosphere soil. *Phytopathology* **82:** 652–656.

Kapsalis AV; Gravanis FT; Gowen SR (2003). Seed treatment with a bacterial antagonist for reducing cotton damping-off caused by *Pythium* spp. *Proceedings of the BCPC International Congress – Crop Science and Technology*. **1**, 655-658. Glasgow, UK.

Vagelas IK; Kapsalis AV; Gravanis FT; Gowen SR (2004**).** Biological control of *Rhizoctonia solani* damping-off with a bacterium symbiotically associated with *Steinernema abbasi*. *International Organization for Biological Control* (IOBC/WPRS) Bulletin 27(1): 285-290.

The effect of previous rearing of *Trichogramma brassicae* on factitious hosts on its acceptance to target hosts

G Nouri-Ganbalani, N Vaez
Plant Protection Dept , Mohagheghe Ardabili University, Ardabil (PO Box 179), Iran, Email: gadirnouri@yahoo.com

S H Iranipour
Plant Protection Dept, Tabriz University, Tabriz (PO Box 5166616471), Iran

M M Jafarloo
Pest & Diseases Research Institute of Tabriz(PO Box 53555-141), Iran

Introduction

The efficacy of biological control agents is dependent on the quality of the natural enemies and it requires the availability of factitious hosts capable of maintaining the potentialities of biological control agents for many successive generations. After several generations of laboratory rearing, it is possible that genetic, physiological, ecological and behavioral potential of these agents regress, which it will affect in turn, the success of biological control. Therefore this research was conducted to study the effect of rearing hosts on the acceptance of the target host.

Methods

All experiments were carried out under 25±1ºC, 65±10% RH, and 16L:8D photoperiod in an incubator. The mated females of the same age of *Trichogramma brassicae* were kept in individual glass vials (10×160mm), and were fed with honey (20%). Fifty to 70 fresh eggs of *Sitotroga cerealella*, *Anagasta kuehniella* (factitious hosts) and *Helicoverpa armigera* (target host) were offered daily to each female until the last one died. The following parameters: sex ratio, fecundity, longevity, emergence rate, r_m (intrinsic rate of natural increase), R_0 (net reproductive rate), T (mean generation time), DT (doubling time) and λ (finite rate of increase) were measured after rearing *T. brassicae* on eggs of *A. kuehniella* Zell. and *S. cerealella* Oliv. for the first two generations separately. Then the same parameters were evaluated after rearing on eggs of *H. armigera* Hüb. for the next two generations, after rearing on factitious hosts separately.

Results

Quality parameters (sex ratio, fecundity, emergence rate and longevity)

Fecundity and emergence rate of *T. brassicae* on *S. cerealella* (S), and *H. armigera* (SH), were significantly different ($F=12.53$, d.f. $=3,70$, $P<0.05$). Sex ratio and female longevity didn't show any significant differences. There were no significant differences in fecundity, sex ratio and emergence rate of adults on *A. kuehniella* (A), and *H. armigera* (AH). The female life time on these hosts showed significant difference ($F=2.92$, d.f. $=3,60$, $P<0.05$) (Table1).

Fertility life table parameters (r_m, R_0, T, DT and λ)

There were no significant differences in mentioned parameters on *A. kuehniella* (A), and *H. armigera* (AH). The mean of fertility life table parameters for two generations (1st & 2nd generation) on eggs of *S. cerealella* (S) were 0.3895 (r_m), 1.4763(λ), 62.31 (R_0), 1.778 (DT) and for the next two generations (3rd & 4th) on eggs of *H. armigera* (SH), were 0.3476 (r_m),

1.4158 (λ), 44.69 (R_0), 1.994 (DT), that showed significant differences (F=0.2139, d.f. =2, P<0.05). The mean generation time on these hosts didn't show any differences (Table2).

Table1. Sex ratio, fecundity, emergence rate and longevity of *T. brassicae* on different hosts

	Mean ± SE			
Parameter	A	AH	S	SH
sex ratio	0.83±0.02ab	0.85±0.01a	0.79±0.02b	0.82±0.02ab
fecundity	60.71±4.51b	51.2±3.41b	93.0±6.43a	64.26±4.06b
emergence rate	0.94±0.005ab	0.93±0.004b	0.94±0.002a	0.91±0.003c
longevity	7.42±0.52b	9.0±0.5a	8.12±0.45ab	8.98±0.43a

Table 2. r_m, R0, T,DT and λ of *T. brassicae* on different hosts

		Mean ± SE				
Tr.	n	rm	Ro	T	DT	λ
A	45	0.3549±0.006b	42.47±2.90a	10.54±0.12a	1.95±0.03a	1.426±0.009b
AH	50	0.3417±0.007b	37.57±2.54b	10.62±0.10a	2.03±0.04a	1.407±0.01b
S	50	0.3895±0.006a	62.31±3.76a	10.61±0.13a	1.78±0.03b	1.476±0.009a
SH	49	0.3476±0.005b	44.68±2.35b	10.87±0.09a	1.99±0.03a	1.416±0.007b

Discussion

Van Bergeijk *et al.* (1989) reported that *Trichogramma maidis* had reduced acceptance to its natural host eggs of the European corn borer, after three generations of continuous rearing on eggs of the *A. kuehniella*. Hassan (1989) also reported that 17 strains of *T. dendrolimi* had the highest fecundity and showed a nearly equal preference between the target pests *Cydia pomonella*, *Adoxophyes orana* and the factitious host (*S. cerealella*) after rearing for at least two generations. On the other hand, the capacity of *T. brassicae* to parasitize eggs of target host was significantly influenced by the rearing hosts (Roriz, *et. al.* 2006). The finding of this study suggested that host acceptance of *T. brassicae* to *H. armigera* as a target host did not change after rearing on *A. kuehniella* as a factitious host. Also, we observed that *T. brassicae* parasitize the factitious hosts more than the target host. This difference can be related to adaptation of these species to the hosts used to rear.

References

Bergeijk K E van; Bigler F; Kaashoek N K; Pak G A (1989). Changes in host acceptance and host suitability as an effect of rearing *Trichogramma maidis* on a factitious host. *Entomologia Experimentalis et Applicata* **52,** 229–238.

Hassan S A (1989). Selection of suitable Trichogramma strains to control the codling moth *Cydia pomonella* and the two summer fruit tortrix moths *Adoxophyes orana*, *Pandemis heparana* (Lep.: Tortricide). *Entomophaga* **34,** 19–27.

Roriz V; Oliveira L.; Garcia P (2006). Host suitability and preference studies of *Trichogramma cordubensis* (Hym.: Trichogrammatidae) *Biological Control.* **36,** 331-336.

Breeding and application of the natural enemy *Scleroderma guanica* to control pests of medicinal plants

J Chen, H Cheng, J Yu, R Xu, S Chen
Institute of Medicinal Plant, Chinese Academy of Medical Sciences, Beijing 100094, China
Email: jchen@implad.ac.cn, junzichen@263.net

Introduction

There are about 11, 000 plants with known medicinal properties and more than 200 of these are grown commercially. Pests affect both wild and cultivated medicinal plants. Using pesticides to control pests is a common practice but misuse of pesticides can result in high residue levels in medicinal plants. Every year there have been substantial economic losses in production of Chinese medicinal plants as a result of diseases and insect pests. Pesticide residues above the standard limits have become an obstacle to the progress of Chinese medicinal plants to the market.

A major pest of medicinal plants is the wood borer. This can be avoided by use of natural enemies. The natural enemy *Scleroderma guanica,* an ectoparasitic insect found in China, has proved to be effective in the control of pests of medicinal plants.

Scleroderma guani is a natural enemy of Hymenoptera and Bethyloidea. These bees are small but have a strong boring ability. The females have some special advantages, such as longevity, fertility, a wide host range and the females' reproduction coefficient is high. According to preliminary statistics, guani can parasitize Lepidoptera, Coleoptera, Hymenoptera and longhorn beetles, in all about 70 species. These bees survive over winter, both in nature and when laboratory bred, allowing continued control of the pest. They have been particularly effective for pests which are difficult to control. Therefore, guani has great potential as a natural enemy of insects.

Currently, guani is bred from host borers collected from the wild forests. Although this breeding method has good effect, it is difficult to collect the host. Also, production of the natural enemy is restricted by the natural season of host reproduction, the natural location and the lack of control of quantity. For production to meet market demand for the natural enemy *Scleroderma guani*, we launched a study aimed at using intermediate host to breed guani.

The study and the results

The results clearly showed that use of a suitable intermediate host can increase the parasitism significantly. Studies on the relation of host growth and parasitism, using 'Uniform Design regression analysis and optimization system', showed that some treatments in the host can increase the rate of parasitism. Different species and ages of *S. guanica* can differ in parasitism.

The biological control agent maintains efficacy for 10 months, compared to two to three months without an intermediate host. The storage life of the natural enemy has been increased from 20-40 days to longer than 200 days using appropriate storage preservation technology.

Borers from Guangxi, Yunnan, Hainan and Zhejiang provinces of China were investigated in woody and vine medicinal plant in order to identify the species present in these areas. Laboratory tests showed that *Scleroderma guanica* can parasitise the following borers: *Apomecyna saltator* (Fabrici us), pest of *Momordica grosvenori*; *Acalolepta sublusca* (Thomson) pest of *Tripterygium wilfordii*; *Oberea fuscipennism holoxantha* Fairm, pest of Rauwolfis and also *Hylotrupes bajulus* which is a storage pathogen. Parasitism of up to 56% was achieved with *Hylotrupes bajulus*. A field survey showed the parasitic rate of *Apomecyna saltator* is between 44.5% and 65.3%.

The results achieved testify that *Scleroderma guanica* can result in good pest control and may be of particular value in controlling pests of medicinal plants.

Utilization of green lacewing, *Mallada basalis* (Walker) (Neuroptera: Chrysopidae) for augmentative biological control of thrips in asparagus in Thailand

O Kern-asa, W Suasa-ard, S Uraichuen
National Biological Control Research Center, Central Regional Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand
Email: agropk@ku.ac.th

Abstract

The augmentative biological control of thrips in asparagus fields were conducted in three locations at Kamphaeng Saen district in Nakorn Pathom, Thailand. This insect pest is one of the important key pests of asparagus which causes serious damage to asparagus yields either in quantity or quality. Lacewings were mass reared in the laboratory and then separately liberated in asparagus fields as egg releasing and neonate larvae releasing, respectively. Counting of thrips present in release plots and conventional plots taken care by farmers, showed that thrips populations in treated plots were significantly decreased compared to control plots. At the same time, there was difference between utilization of eggs and neonate larvae of lacewing to control thrips since the percentage of control ranged from 65 to 75 percent. This indicated that green lacewing can be further recognized as biological agent to control thrips for asparagus production in Good Agricultural Practice (GAP) under which insecticide was unacceptable during the production period.

Introduction

Asparagus production in provinces nearby Kasetsart University, Nakhon Pathom, Thailand is carried out under GAP where insecticides are unacceptable during the harvesting period. Biological control is then obligatory as an alternative. *Thrips tabaci* and *T. palmi* are serious insect pests of many crop plant species including asparagus. Direct feeding damage to leaves, flowers and fruit caused by the ingestion of sap is most harmful in dry climates and seasons. The infestation can seriously deplete yields and sometimes render crops uneconomic (Lewis, 1997). The green lacewing, *M. basalis,* is an important predator which can reduce several insect pests and mites such as thrips, aphids, whiteflies and mealybugs (Chandish and Singh, 1999).

Materials and methods

Utilization of *M. basalis* for control of *Thrips* spp. was conducted in farmer's asparagus fields. The experiments were done in three plots composed of two release plots and one non-release plot at Kamphaeng Saen, Nakhon Pathom province. The two release plots were compared for the use of eggs and neonate larvae for control of *Thrips* spp.; 5,000 of each stage were released per acre in each plot in weekly intervals. The number of thrips present in released and non-released plots was counted by random sampling method with 50 samples (one shoot/plant was one sample) every week before the releasing date and were then compared.

Results and discussion

The on-farm counting of *Thrips* spp. and *M. basalis* larvae populations revealed that the population of *Thrips* spp. was decreased in three plots. In plots in which either eggs or neonate larvae of *M. basalis* were released, there was a 50.53% and 68.49% decrease of *Thrips* spp., respectively, while in non-release plot, there was only 37.02% decrease inbetween 4 August and 1 September (Table1).

Table 1 Population density of *Thrips* spp. in release plots utilizing eggs and neonate larvae of *M. basalis* compared to non-release plots

Treatments	Population density of *Thrips* spp. / shoot					% decreased
	4 Aug 2006	11 Aug 2006	18 Aug 2006	25 Aug 2006	1 Sep 2006	
Egg- release plot	9.4±5.22	6.6±3.02	6.25±2.36	5.8±2.36	4.65±1.85	50.53
Neonate larvae release plot	7.3±3.39	5.0±2.04	3.9±1.76	3.35±1.06	2.3±0.64	68.49
Non-release plot	10.4±2.84	10.2±2.63	8.85±3.55	8.15±3.72	6.55±4.8	37.02

This experiment indicated that release of neonate larvae of *M. basalis* was better than egg-release similar to the study of Daane & Yokota (1997) which reported that *C. carnea* as neonates reduced the population of leafhopper ranging from 0-36.7% in vineyards. Horne, *et al*. (2001) equally reported that *M. signatus* larvae were more voracious on *H. punctigera* larvae in cotton fields in Queensland compared to egg release. In conclusion, *M. basalis* was considered suitable as biological control agent to control thrips for asparagus production in GAP in Thailand.

References

Candish R B; Singh S B (1999). Host plant-mediated orientational and ovipositional behavior of three species of Chrysopidae (Neuroptera: Chrysopidae). *Biological Control*. **16,** 47-53

Daane K M; Yogota G Y (1997). Release strategies affect survival and distribution of green lacewings (Neuroptera: Chrysopidae) in augmentative programs. *EnvironmentalEntomology*. **26(2)**, 455-464

Horne P A; New T R; Papacek D (2001). Preliminary notes on *Mallada signatus* (Chrysopidae) as a predator in field crops in Australia. In: *Lacewings in the Crop Environment*. McEwen, *et al* (eds.). Cambridge University Press. UK. pp. 395-397

Lewis T (1997). *Thrips as Crop Pests*. CAB International, Wallingford, UK, 40 pp.

Utilization of larval parasite *Cotesia flavipes* for augmentative biological control of sugar cane moth borers in Thailand

W Suasa-ard, K Suksen, O Kernasa
National Biological Control Research Center, Kasetsart University, Bangkok, Thailand
Email: agrwis@ku.ac.th

Introduction

Cotesea flavipes is considered as the most effective larval parasite of sugarcane moth borers, *Chilo infuscatellus*, *Chilo sacchariphagus*, *Chilo tumidicostalis* and *Sesamia inferens*. These borers cause economic damage in every cane growing area (Suasa-ard, 1982). Interest in the use of *C. flavipes* for controlling sugarcane moth borers has increased in many countries such as Brazil, Jamaica, India, Mauritius, Pakistan and South Africa (Mohyuddin, 1992). The purpose of this investigation was to evaluate the utilization of *C. flavipes* for augmentative biological control of sugarcane moth borers.

Materials and methods

Mass-rearing of *C. flavipes* was carried out at the National Biological Control Research Center-Central Regional Center, Nakhon Pathom, Thailand. By using the technique given by Suasa-ard & Permniyomkit (2000). Nakhon Pathom, Supan Buri and Kanchanaburi provinces in the Central Region of Thailand were selected as investigation sites. At each site two plots (ca. 20 acres) were selected as release and control plots. About 3000 adults of *C. flavipes* were released at monthly intervals from March to December in 2003, 2004 and 2005. Field assessment in release and control plots at each location was investigated.

Results and discussion

Field assessment of *C. flavipes*

Comparison between percent parasitization of sugarcane moth borers by *C. flavipes* in release and non-release plots at every location were evaluated. It was obvious that the parasitization in release plots were higher than those in non-release plots. The average percent parasitization in release plots at Nakhon Pathom, Suphan Buri and Kanchanaburi province were 23.38, 20.19 and 26.64 respectively in 2004 (Table 1). Assessment infestation to sugarcane caused by the sugarcane moth borers confirms the success of *C. flavipes* in controlling the population of sugarcane moth borers. The percent infestation of sugarcane is illustrated in Table 2. It reveals that after parasites were released, infestation levels decreased with a low peak occurring from July to September at every location. The lowest average percent infestation in release plots at Nakhon Pathom, Suphan Buri and Kanchanaburi province were 6.62, 6.24 and 6.28 respectively in 2004. This was the same as reported by Suasa-ard *et al.* (2001).

Conclusions

The inoculative release of *C. flavipes* every month effectively controlled the sugarcane moth borer. The percent parasitization and percent infestation of sugarcane moth borers in release plots were significantly different than in non release plots at every location. The differences show the efficacy of *C. flavipes* as biological control agent of sugarcane moth borer in Thailand.

Table 1. Average percent parasitization of sugarcane moth borers by *C. flavipes* in release and control plots during 2003 to 2005.

Location	Percent parasitization (%)					
	2003		2004		2005	
	Mean (%)	range (%)	Mean (%)	range (%)	Mean (%)	range (%)
Nakhon Pathom						
Release plot	15.99c	1.0-24.6	23.38ab	5.4-30.5	13.26c	4.08-21.43
Control plot	5.3d	0.4-8.9	4.03d	1.2-6.1	4.30d	2.38-5.71
Suphanburi						
Release plot	18.05bc	4.3-25.7	20.19bc	8.2-28.9	11.96c	0.93-21.87
Control plot	5.58d	0.9-8.5	3.73d	2.1-5.2	2.25d	0.91-4.65
Kanchanaburi						
Release plot	22.27ab	4.2-30.5	26.64a	10.9-38.6	13.30c	5.83-23.33
Control plot	3.89d	2.3-5.9	3.84d	1.6-6.4	6.70d	4.54-9.09

Means followed by different letter were significantly different at 95%

Table 2. Average percent infestation of sugarcane moth borers in release and control plots.

Location	Percent infestation (%)					
	2003		2004		2005	
	Mean(%)	range(%)	Mean(%)	range(%)	Mean(%)	range(%)
Nakhon Pathom						
Release plot	7.18e	4.5-11.4	6.62e	3.1-9.8	15.57b	8.00-21.57
Control plot	11.00de	8.5-15.6	11.38c	9.3-14.3	28.12a	20.24-39.16
Suphanburi						
Release plot	7.87de	3.5-18.4	6.24e	2.9-14.6	15.28b	10.00-23.36
Control plot	14.83b	7.8-20.2	11.67c	10.7-18.7	29.32a	22.14-34.00
Kanchanaburi						
Release plot	6.39e	4.4-12.1	6.28e	2.8-11.6	13.88b	6.00-24.76
Control plot	14.88b	9.8-18.9	15.51b	8.6-26.5	31.07a	23.16-34.00

Means followed by different letter were significantly different at 95%

References

Mohyuddin AI (1992). Utilization of natural enemies for the control of insect pest of sugarcane. *Biocontrol New and Information* **13**: p 99.

Suasa-ard W (1982). Ecology of sugarcane moth borer and their parasites in Thailand. Ph.D. Dissertation. Kasetsart Univ., Bangkok. 169 p.

Suasa-ard W & Permniyomkit J (2000). Mass-rearing of *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae). In: *Sugarcane Pest Management in the new millennium. Proceedings IV ISSCT Sugarcane Entomology Workshop*. (Eds. PG Allsopp & W Suasa-ard). p. 18 Khon Kaen, Thailand.

Suasa-ard W, Charernsom K, Permniyomkit J & Kernasa O (2001). Augmentative biological control of sugarcane moth borer Chilo tumidicostalis in Thailand. In: *Proceedings XXIV ISSCT Congress* (Ed. DM Hogarth), p.404, Brisbane, Australia.

Production of *Bacillus thuringiensis* biopesticides using a commercial lab medium and agricultural by-products as nutrient sources

F H Valicente, M I S Leite, F L Freire , C M Vieira
Embrapa Maize and Sorghum Research Center, 35701-970, Sete Lagoas, MG, Brazil
Email: valicent@cnpms.embrapa.br

Introduction

Fall armyworm, *Spodoptera frugiperda*, is the most important maize insect pest in Brazil. Its damage can reduce grain production up to 34%. Currently, control of this insect in Brazil is exclusively with chemicals. *Bacillus thuringiensis* (*Bt*) has potential as a cheap agent to be introduced in insect pest management. *B. thuringiensis* can be cultivated using liquid, solid and semi-solid media rich in carbon, nitrogen and mineral salts. Commercial lab media, e.g. Luria Bertani medium (LB) and agricultural by-products (e.g. glucose, soybean flour and liquid swine manure) may be used as nutrient sources to grow *Bt*. The objective of this research was to test LB-medium enriched with salts ($FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$) and agricultural by-products such as maize glucose, soybean flour and liquid swine manure (LSM) as medium for the production of *Bt*.

Materials and methods

Bacillus thuringiensis sv *tolworthi*, strain 344, was obtained from Embrapa Maize and Sorghum Microbial Bank. It was sub cultured in LB medium enriched with salts (0.002 g of $FeSO_4$, 0.02 g of $ZnSO_4$, 0.02 g of $MnSO_4$, 0.3 g of $MgSO_4$ and 2 g of glucose per liter) and the pH was adjusted to 7.5. After sub-culturing, strain 344 was streaked on sporulation medium containing the same mineral salts plus 12g/l of Bacto-Agar (Difco) and 8 g/l of nutrient broth. The plates were incubated for 24 hours at 30±1 °C and maintained at 4 °C for future use. Medium 1 was composed of LB medium plus salts ($FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$), and 0.2 % glucose; medium 2 was composed of 1.5 % glucose and 0.5 % soybean flour plus salts; medium 3 was composed of LSM at 4 %. The latter was collected after 32 days of fermentation, when the C : N ratio was considered to be stable. LSM was diluted to 4 % and enriched with 1.5 % soybean flour and 0.5 % glucose. All three media were sterilized at 121 °C for 30 minutes. 500ml Erlenmeyer flasks containing 150 ml of sterilized media 1, 2 and 3 were inoculated with a seed culture of strain 344. The flasks were incubated on a rotary shaker for 96 h at 30 °C. Samples were drawn at different intervals. For determination of numbers of viable spores the samples were heat-treated (see below) before they were diluted and plated out. For determination of numbers of viable cells they were diluted and plated out directly.

For determination of cell mass, samples of 100 ml were centrifuged at 10.000 rpm for 20 minutes. The supernatant was discarded and the pellet was lyophilized. Dry weight was calculated and expressed in gram per liter. The same sample was used for the spore count and toxicity test. For assessment of spore counts, samples of the cultures were heat-treated at 80 °C for 15 minutes, serially diluted and plated on LB medium amended with salts and glucose. The plates were incubated at 25±1 °C for 24 hours to form fully developed colonies that were counted and expressed in colony forming units per ml (c.f.u./ml). The pH of all media was adjusted to 7.2±0.1 with NaOH after sterilization, and measured at regular intervals during the 96 hours of the fermentation process. The spore crystal complex produced in different media was assayed against two day old larvae (first instar) of *S. frugiperda* raised in the laboratory on an artificial diet. The bioassays were conducted

using pieces of corn leaves (approx. 9 cm^2) washed once with 0.5% sodium hypochlorite and two times with distilled water. Samples of strain 344 produced in the three different media were prepared in sterile distilled water, and 18 µl of each suspension was placed on the corn leaf surface and allowed to dry. The corn leaf pieces thus prepared were fed to the larvae. Each bioassay included seven doses of two replicates each, along with the appropriate control. Larval mortality was scored up to four days after larvae infection.

Results

Since there was no pH control during the experiments, the pH varied during the 96 hours of fermentation. In the commercial LB medium (1), the pH rapidly increased in the first 5 hours of fermentation and quickly decreased to 6.44 after 12 hours of fermentation. However, after this period LM medium was the only one that maintained the pH above 8. Media 1 and 2 showed a tendency to shift toward a basic pH, and medium 3 (LSM) to an acidic pH. Medium 1 stabilized a pH around 8, whereas medium 3 stabilized around pH 3. The yield of spores in medium 1 (LB + salts + glucose) was in the order of 1.7×10^9 spores/ml (2.0×10^8 c.f.u./ml) after 96 hours of fermentation. The yield of spores in medium 2 (maize glucose + soybean flour) was in the order of 3.1×10^8 spores/ml (2.0×10^8 c.f.u./ml) after 96 hours and medium 3 (LSM at 4%) yielded spores in the order of 2.6×10^8 spores/ml (1.5×10^8 c.f.u./ml) after 96 hours. Biomass dry weight of medium 2 (maize glucose + soybean flour) reached its peak producing 1.18 g/l, and LSM at 4 % produced the lowest amount of cell mass of 0.32 g/l. Medium 1 (LB plus salts) produced 1.01 g/l. All three media showed a decrease in cell mass production between 48 and 72 hours of fermentation. Usually, liquid swine manure contains carbon, nitrogen, phosphorus, potassium and solids that vary with their origin. LSM also contains toxic metals (Cu, Zn and Mn) but their concentration was at an acceptable level. LSM is generally a very good source of carbon, nitrogen, phosphorus and other nutrients that support growth, sporulation and endotoxin production by *B. thuringiensis* var. *tolworthi*. The presence of some toxic elements apparently did not affect the final viable cell and spore counts. The largest number of c.f.u. occurred after 96 hours of fermentation. Mortality tests with first instar fall armyworm larvae showed that *Bt* from all three media in the highest concentrations killed above 95 % of the larvae.

Conclusions

It is important to stress that the raw materials used for the production of *Bt*–based microbial pesticides represent a substantial part of the overall production cost. Therefore, it is important to find less expensive by-product sources than those currently used. The use of maize glucose, soybean flour and liquid swine manure represents a new alternative for disposal and/or recycling of these substrates and decreases the production costs for *Bt*-pesticides. Our results indicate that maize glucose, soybean flour and liquid swine manure contain the necessary nutrient elements to grow *B. thuringiensis tolworthi.*

The use of plant essential oils for the control of pine wood nematode (*Bursaphelenchus xylophilus*)

I K Park, J H Kim, S G Lee, Y S Lee, S C Shin
Division of Forest Insect Pests and Diseases, Korea Forest Research Institute, Seoul 130-712, Korea
Email: parkik1@foa.go.kr

Introduction

Pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus*, is a serious problem in southern Korea. The disease was first reported in Busan city in 1988 (Yi *et al.*, 1989) and has spread to several southern and middle areas of the Korean peninsula. As *Pinus densiflora* and *P. thunbergii* are predominant tree species in Korean forests and are very susceptible to the pinewood nematode, ecological and economical damage is substantial. Control of this disease depends on fumigation of disease-infected trees, aerial application of synthetic pesticides against *Monochamus alternatus,* the insect vector of this disease, or injection of nematicides (Korea Forest Service, 2003). However, human health concerns regarding commercial nematicides, factors such as increased cost of nematicide, labour and application have made pine wilt disease control difficult and so alternative control methods or less toxic nematicides need to be developed. Plant essential oils are potential alternatives to current control agents. They constitute a rich source of bioactive chemicals, and are commonly used as fragrances and flavouring agents for foods and beverages (Isman, 2000). We investigated plant essential oils and their components which have nematicidal activity against pine wood nematode.

Nematicidal activity of plant essential oils

When 43 plant essential oils were bioassayed, essential oils of *Cymbopogon citratus, Cinnamomum verum* (cinnamon), *Allium sativum* (garlic), *Leptospermum petersonii* and *Eugenia caryophyllata* caused 100% immobility. *Asiasarum sieboldi* and *Mentha spicata* produced 100% mortality of male and female nematodes, but 78.3 and 85.5 % mortality of juveniles, respectively. The toxicity of *Boswellia carterii* and *Pimenta racemosa* was approximately 70-80%. The other plant essential oils showed weak or no activity. Plant essential oils showing good activity were bioassayed at lower concentrations. Garlic oils produced 100% mortality at 62.5 $\mu l\ l^{-1}$ against male, female and juvenile nematodes but these decreased to 74.7, 77.7 and 76.9% at 31.25$\mu l\ l^{-1}$, respectively. The toxicity of *Cinnamomum verum* was very high at 125 $\mu l\ l^{-1}$, but gave moderate activity at 62.5 $\mu l\ l^{-1}$. The other plant essential oils produced strong activity at higher concentrations (1000 or 500 $\mu l\ l^{-1}$) but weak or no activity was observed at lower concentrations. To identify the active compounds of garlic and cinnamon oils, we used GC-MS. Main components of garlic oil were diallyl sulphide (21.3%), diallyl disulphide (59.7%) and diallyl trisulphide (10.9%). Cinnammaldehyde (78.4%) and cinnamyl acetate (5.7%) were identified from cinnamon oil. The toxicity of identified compounds is shown in Table 1. The compound most toxic to the pinewood nematode was diallyl trisulphide followed by cinnamyl acetate, diallyl disulphide, cinnamaldehyde and diallyl sulphide. The LC_{50} values of diallyl trisufide were 3.72, 2.79 and 2.79 $\mu l\ l^{-1}$ against males, females and juveniles, respectively. Juveniles were the most susceptible (LC_{50} = 32.81$\mu l\ l^{-1}$), and males were the most resistant (LC_{50} = 39.30$\mu l\ l^{-1}$) against cinnamyl acetate. In a test with diallyl disulphide, LC_{50} values were 43.20, 46.48 and 37.06 $\mu l\ l^{-1}$ against male, female and juvenile nematode, respectively.

Table 1. Toxicity of constituents from garlic and cinnamon oils against *B. xylophilus*

Compounds	LC_{50} ($\mu l\ l^{-1}$)		
	Males	Females	Juveniles
cinnamyl acetate	39.30 (32.33-47.51)[1]	36.50 (29.71-44.33)	32.81 (26.08-40.30)
diallyl disulphide	43.20 (35.33-52.75)	46.48 (38.44-56.31)	37.06 (29.84-45.44)
diallyl trisulphide	3.72 (3.11-4.51)	2.79 (2.24-3.48)	2.79 (2.23-3.51)

[1]Values in parentheses indicate 95% confidence limit

Nematicidal activity of monoterpenoids

A large number of plant compounds called isoprenoids are formed by the condensation of five-carbon isoprene units. Among the simplest are 10-carbon compounds called monoterpenoids, which are major components of plant essential oils. Monoterpenoid compounds have been considered as potential pest control agents because they are acutely toxic to insects and possess repellent activity. Monoterpenoids were selected in this study because many plant essential oils have proved to be active against the pine wood nematode (Park et al., 2005). A total of 26 monoterpenoids were selected to investigate the nematicidal activity against pine wood nematode. When 26 monoterpenoids were bioassayed, phenols such as carvacrol and thymol showed the most potent activity against the pine wood nematode. LC_{50} values of carvacrol against male, female and juvenile nematode were 0.125, 0.097 and 0.099 mg/ml, respectively. Among the alcohol group, citronellol showed the most potent activity. Juveniles were more susceptible (LC_{50} = 0.169 mg/ml) than males (LC_{50} = 0.245 mg/ml) and females (LC_{50} = 0.235 mg/ml). Toxicity of geraniol was weaker than citronellol. LC_{50} values of geraniol against male, female and juveniles were 0.540, 0.415 and 0.417 mg/ml, respectively. Nerol and menthol had similar LC_{50} values. The other alcohols displayed weak activity. In the aldehyde group, citral showed the strongest activity. LC_{50} values of citral against male, female and juveniles were 0.187, 0.139 and 0.110 mg/ml, respectively. LC_{50} values of citronellal against male, female and juveniles were 0.321, 0.298 and 0.253 mg/ml, respectively. LC_{50} values of all hydrocarbons were $>$1.0 mg/ml.

Conclusion

Our results indicate that plant essential oils and their components could be useful as nematicides for use against the pinewood nematode. Further study is necessary to assess the safety of these materials to humans and to develop formulations to improve their efficacy and stability and to reduce their cost before they are implemented as a control measure.

References

Isman, M B (2000). Plant essential oils for pest and disease management. *Crop Protection* **19**, 603-608.

Korea Forest Service. (2003). *Guideline for the control of forest diseases and insect pests.* Korea Forest Service, Daejeon, Republic of Korea, 198 pp.

Park I K, Park J Y, Kim K H, Choi K S, Choi I H, Kim C S and Shin S C, (2005). Nematicidal activity of plant essential oils and components from garlic (*Allium sativum*) and cinnamon (*Cinnamomum verum*) oils against the pine wood nematode (*Bursaphelenchus xylophilus*). *Nematology* **7**, 767-774.

Yi C K, Byun, B H Park, J D, Yang, S I and Chang K H, (1989). First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and its insect vector in Korea. *Research Reports of Forestry Research Institute* **38**, 141-149.

Isolation, identification and activity of insecticidal components from *Streptomyces* sp. 4138

B R Lin, X M Pu, H F Shen
Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, Wushan, Guangzhou, China
Email: Linbr@126.com

M Y Hu, X G Li
South China Agricultural University, Guangzhou, 51064 China

Introduction

In order to develop new biocontrol agents, soil samples were collected from all over the world, and 2200 actinomycete strains were isolated. In bioactivity screenings it was found that 13 strains had insecticidal activity. The most effective strain was *Streptomyces* sp. 4138. We here report the fermentation of *Streptomyces* sp.4138, as well as the isolation and identification of the biologically active component and its activity on *Spodoptera exigua.*

Materials and methods

Streptomyces sp. 4138 was cultured in a 30 liter fermentation jar at 28°C for 96h. After fermentation, the mycelium was extracted with ethyl acetate and n-butyl alcohol. The extraction gradient was eluted with different proportions of methanol/chloroform 2L in vacuum liquid chromatography (silica gel H, 100~200). The active component was purified by preparative HPLC, collected and freeze-dried in order to identify the structure. Bioactivity of staurosporine was characterized on third stage larvae of *S. exigua*. The parameters evaluated included peroral toxicity, contact toxicity, antifeedant activity, and influence on growth, development and reproduction.

Results

The active compound could be dissolved in DMSO and ethyl acetate but not in water. The signals of ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMBC, DEPT and HSQC spectra showed that the insecticidal component was staurosporine. The bioactivities of staurosporine were peroral toxicity and antifeedant activity. After 48 h exposure the peroral toxicity (LC_{50}) was 5.0 μg/ml, and the antifeedant activity (AFC_{50}) 1.1 μg/ml (Table 1).

Table 1: Activity against third stage larvae of *S. exigua*

Treatments	24h	48h
Peroral toxicity (LC_{50}) (μg/ml)	6.4	5.0
Antifeedant activity (AFC_{50}) (μg/ml)	2.5	1.1
Contact toxicity (LC_{50}) (μg/ml)	26.5	20.3

The sensitivity towards staurosporine depended on the larval stage used, with later larval stages being less sensitive. The concentration of staurosporin and the pupation rate were inversely correlated, i.e. the higher the staurosporin concentration, the lower the pupation rate. However, pupa weight and emergence rate were not significantly affected by staurosporine (Table 2).

After intake of staurosporine, the number of eggs deposited by adults was reduced compared to feeding on water or on 10 honey. It can be inferred that staurosporine has an effect on the population size of the next generation of *S. exigua* (Table 3).

Table 2: Effect of staurosporin on pupation, pupa weight and emergence of third stage larvae of *S. exigua*

Concentration of staurosporine (µg/mL)	Pupation rate (%)	Pupa weight (mg)	Emergence rate (%)	Sex ratio (♂/♀)
4.0	28.9 ±1.11 d	85.5 ±6.2 a	92.6 ±3.70 a	0.5 ±0.07 b
2.0	42.2 ±4.01 c	85.6 ±2.9 a	90.0 ±5.09 a	0.9 ±0.38 a
1.0	45.6 ±2.94 c	91.1 ±6.7 a	92.6 ±0.49 a	1.2 ±0.39 a
0.5	63.3 ±3.33 b	92.2 ±2.2 a	91.3 ±1.65 a	1.1 ±0.37 a
Control	80.0 ±5.77 a	100.1 ±5.2 a	93.0 ±1.56 a	0.6 ±0.06 b

Table 3: Influence on longevity of adults and egg deposition of *S. exigua*

Concentration of staurosporine (µg/mL)	Fecundity	Female adult longevity (days)	Male adult longevity (days)
10.0	26.0 ±6.27 e	4.3 ±0.26 d	3.5 ±0.13 d
5.0	76.0 ±14.20 d	5.0 ±0.35 c	4.0 ±0.20 cd
2.5	184.0 ±12.72 c	5.4 ±0.24 c	4.6 ±0.32 c
water	309.6 ±22.11 b	6.2 ±0.16 b	5.6 ±0.24 b
10 honey	625.2 ±29.11 a	8.4 ±0.16 a	7.4 ±0.44 a

Discussion

Antibiotic insecticides have been studied since the 1950s. However, the subject was ignored at that time because most researchers in insect control tended to apply highly effective and hypertoxic chemical pesticides such as DDT, but paid little attention to insecticidal antibiotics. However, with the increasing awareness of environmental problems, antibiotics became a new attraction to pesticide scientists since the successful development of avermectins by Beili Institute of Japan and Merck of the United Sates in 1975.

Staurosporine is an alkaloid compound discovered in 1987. It was found to be insecticidal to *S. exigua* in USA, but no further research has been done since then. *S. exigua* is one of the main insects pests of crops and vegetables. In China it is resistant to many types of pesticides. We therefore analyzed the effect of straurosporine on *S. exigua*. From a pot experiment it was concluded that staurosporine has high biological activity against *S. exigua*. Staurosporine may be developed to a highly active and safe pesticide for control of *S. exigua*.

A systemic bioinsecticide containing azadirachtin for control of an invasive woodboring beetle, the emerald ash borer, *Agrilus plannipennis*

B V Helson, D G Thompson
Natural Resources Canada, Great Lakes Forestry Centre, 1219 Queen St E., Sault Ste. Marie, Ontario P6A 2E5, Canada
Email: bhelson@nrcan.gc.ca

G W Otis, N G McKenzie
Dept Environmental Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

J Meating
BioForest Technologies Inc., 105 Bruce St. Sault Ste Marie, Ontario P6A 2X6, Canada

Azadirachtin extracts are known to possess systemic activity on several defoliating and leafmining insects when injected into trees (e.g. Helson *et al*, 2001). A new proprietary, liquid formulation named TreeAzin, containing the safe, natural product, azadirachtin using NeemAzal technical (E.I.D. Parry India Ltd.), has been developed specifically for tree trunk injections. Low volumes of this 5% formulation can be injected quickly and completely into trees with a new, high output, commercial tree delivery method, the EcoJect System. In 2002, the emerald ash borer (*Agrilus planipennis*) (EAB), an invasive woodborer from China, was discovered in Windsor, Ontario, Canada and Detroit Michigan USA. This serious pest has attacked and killed millions of native ash (*Fraxinus*) trees and is continuing to spread in both Canada and the USA.

In an effort to find a solution to protect high value ornamental ash trees from damage by EAB, the potential of management using azadirachtin was assessed by determination of (1) uptake, translocation and persistence following trunk injections of ash trees as it relates to magnitude and duration of residues in foliage, (2) efficacy on larvae in small and large trees as measured by changes in adult emergence and ash tree crown density and (3) effects on adult mortality, female fecundity and fertility following ingestion of ash leaves containing azadirachtin. Following injection of 2.2-cm diameter, potted, nursery green ash with 55 mg azadirachtin per cm diameter in June, azadirachtin was rapidly taken up and translocated within the trees. Substantial concentrations (11.4 μg/g dry mass) were present in the canopy of these small trees within seven days of treatment. Concentrations dissipated in a slow curvilinear pattern through a period of 55 days after injection. A similar pattern was observed in larger, 22-cm diameter ornamental trees injected with 250 mg azadirachtin per cm diameter. Estimated DT 50 values were 21 days and 22 days for small and large trees respectively. The data suggest that toxicologically significant concentrations of azadirachtin are likely to occur throughout the majority of the larval and adult feeding period in green ash trees systemically treated with this custom formulation.

For efficacy assessment, 8cm diameter small green ash trees in a plantation and 37-cm diameter large trees on a golf course were injected with azadirachtin at 100 mg per cm diameter (low rate) or 250 mg per cm diameter (high rate) on 28-30 June and 12 July respectively. None of the treated small trees (n=10) at either rate had any new EAB emergence holes compared to 100% of the control trees. The control trees had an average of 12.7 ± 15.5 new emergence holes. The crown densities of the treated small trees were significantly higher than the control trees. Mean crown densities ± SD were 52.5 ± 40.2%

for controls, 94.0 ± 4.6% for the low rate and 88.5 ± 22.7% for the high rate. In the large golf course trees, both emerged adults from and emergence holes on branches collected from the injected trees (n=7) at both rates were significantly reduced by at least 82% compared to untreated trees one year after injection (Table 1). New emergence holes were also significantly reduced on the trunks of the trees treated with the high rate but not the low rate compared to controls. Crown densities of treated trees were higher than controls but differences were not significant. These trees were at an advanced stage of EAB attack at the time of treatment which may explain the relatively poor protection.

Table 1. Emergence holes, adult emergence and crown densities of large ash trees injected with azadirachtin, 12 July 2005.

Rate mg azad. /cm diam.	Branches – adults Mean ± SD	Branches – new emergence holes Mean ± SD	Trunk – new emergence holes Mean ± SD	Crown density Mean % ± SD
0	8.7 ± 12.4 a	11.1 ± 16.5 e	38.6 ± 32.7 s	23.6 ± 25.4 x
100	0.5 ± 1.6 b	2.0 ± 4.1 f	24.3 ± 47.2 s*	37.9 ± 32.1 x
250	0.2 ± 0.5 b	1.4 ± 2.9 f	1.7 ± 4.1 t	49.2 ± 25.0 x
	H = 14.7 P < 0.001	H = 6.7 P = 0.036	H = 10.2 P = 0.006	F = 1.4 P = 0.28

* 1 tree had 130 exit holes.

To evaluate effects on adult mortality, fecundity and fertility, stems of freshly cut green ash leaves were placed in vials containing either distilled water or 5 ppm aqueous solutions of azadirachtin for three days to allow uptake of azadirachtin. The leaves were then transferred to new vials containing fresh distilled water and a pair of male and female EAB was placed on each leaf in a cage to feed for three days. A total of 71 control pairs and 72 treated pairs were set up and observed regularly for mortality and eggs. All eggs were collected and checked regularly for hatching. Azadirachtin did not reduce survival of either males or females over a 30 day period. It also did not reduce the numbers of females laying eggs. However, azadirachtin significantly reduced the fecundity of females by 66% and the fertility of females by 98%.

In conclusion, azadirachtin is rapidly taken up and effectively translocated in ash trees following trunk injections with a 5% formulation which was developed specifically for this purpose. Early summer therapeutic injections at 250 mg azadirachtin per cm diameter provides effective control of EAB larvae for at least one season in both small and large trees. A lower rate of 100 mg per cm diameter is expected to provide acceptable control as aprophylactic treatment or a therapeutic treatment at early stages of infestation. Azadirachtin has no direct effect on the survival of adults but may reduce the fecundity and fertility of females feeding on leaves of injected trees. Trunk injections with TreeAzin offer an effective, highly targeted new tool for the management of emerald ash borer and has great potential for other wood boring beetles.

Reference

Helson BV; Lyons DB; Wanner KW; Scarr TA (2001). Control of conifer defoliators with neem-based systemic bioinsecticides using a novel injection device. *The Canadian Entomologist* **133,** 729-744.

Pasteuria penetrans as a commercial bio-nematicide

I K Vagelas, S V Leontopoulos, F T Gravanis
Technological Education Institute of Larissa, Department of Plant Production, 41110 Larissa, Greece
Email: vagelas@teilar.gr

Root-knot nematodes (RKNs) of the *Meloidogyne* spp. are the most significant plant-parasitic nematodes that damage tomatoes in central Greece (Thessalia region). Apart from the direct damage to roots, *Meloidogyne* spp. enhance wilt disease caused by either *Fusarium oxysporum* or *Verticillium dahliae*. It is well known that the free living second stage juvenile (J2) causes most injury to plants. J2s move in the soil water films to reach the root zone. Therefore, the elimination or halt of J2s into soil could be an import strategy to control root-knot damage. An environmentally friendly approach to control RKNs is to apply the widely known RKN-parasite bacterium *Pasteuria penetrans*. *P. penetrans* (Pp) shows great potential as a biocontrol agent as it is capable of immobilising RKN J2s (Vagelas *et al*., 2006).

A commercial product containing a Pp strain with good attachment to *Meloidogyne* spp., was used to control a mixed population of RKN, consisting of *M. ingognita*, *M. javanica* and *M. hapla,* within a tomato greenhouse. An attachment bioassay was also carried out. One ml of 100±18 freshly hatched J2 per ml water were added into a Petri dish filled with water with 5000 Pp spores per ml. The attached Pp spores were counted after 24 and 48 hours exposure of RKN J2s to Pp. There were significant differences between the two different times of exposure, with an attachment rate of 10-15 spores per J2 after 24 h and >30 spores per J2 after 48 h.

An *in planta* experiment was conducted in order to evaluate nematode development, with or without Pp spores attached. Four week old tomato plants, grown in pots (one plant per pot) were inoculated with 400±32 J2s per plant. Treatments were plants without J2s and Pp spores; plants with J2s only; plants with J2s and 10-15 Pp spores per J2 and plants with J2s encumbered with >30 Pp spores per J2. Treatments were replicated twelve fold. Pots were maintained on a glasshouse bench for 25 days. After incubation, all plants were uprooted, washed under tap water and the number of root galls and RKN egg masses were recorded. The results of the experiment showed that when higher densities of Pp spores were attached to J2s (more than 30 Pp spores per J2) the rate of RKN root infection was greatly reduced (Table 1).

Furthermore, digital image analysis was used to record the effect of Pp spore attachment on the movement of J2s. J2s of *Meloidogyne* spp. with either 10-15 or >30 Pp spores attached or without attached Pp spores, were tracked with an inverted microscope (MICROTEC 200) mounted with a digital photo-camera (AIPTEC 3.2 Megapixel). In all cases, a single nematode's movement was observed in water in a 9 cm Petri dish. The microscope magnification was x100 and the digital camera captured images with 320x240 pixel resolution. After recording, video sequences showing movement of an individual nematode were observed on Movie Maker 2 (Microsoft software). Metrics of nematode movement were performed using image analyzer software Scion Image for Windows (Scion Corporation, www.scioncorp.com).

It was concluded that nematodes without attached Pp spores move further than those with attached Pp spores (Table 2). Nematodes with 10-15 Pp spores per J2 produced positive and negative velocity values, probably because of the forward and backward movement. But nematodes with >30 Pp spores per J2, have zero or almost zero velocity values (Table 2). That means that a high number of Pp spores attached onto a J2 cuticle inhibits J2 movement and probably restricts the ability of the J2 to locate a host root. This is in agreement with the *in planta* experiment data. The overall conclusion of the present study is that the high numbers of Pp spores attached to J2s can halt RKN movement. This result confirms the significantly low galling and egg mass numbers in plants (Table 1).

Table 1. Effect of *Pasteuria penetrans* on nematodes galls/plant and eggmasses/plant.

	Galls		Egg masses	
Treatment	Mean	StDev*	Mean	StDev
Control	0a**	0	0a	0
No Pp	23.75c	10.26	17.25c	11.06
Pp 10-15sp	10. 5b	5.6	7. 5ab	5.2
Pp>30sp	1.75a	1.58	0.13a	0.35
P value	*P<0,001*		*P<0,001*	

Where: Control=Untreated with nematodes plants, No Pp=Plants treated only with nematodes, Pp 10-15sp or Pp>30sp= Plants treated with nematodes with 10-15 Pp spores attached or with >30 Pp spores/J2 attached, respectively. * StDev=the sample Standard Deviation. ** Means within columns followed by the same letter do not differ (p=0.05) according to Tukey's multiple range test.

Table 2. Travel distance [1] of nematodes encumbered with or without *P. penetrans* spores.

Treatment*	Mean*	StDev*
No Pp	2.95 **	0.54
Pp 10-15sp	0.17	0.46
Pp>30sp	0.01	0.03

Where: No Pp= J2s without *P. penetrans* spores attached, Pp 10-15sp or Pp>30sp= J2s with 10-15 or with >30 *P. penetrans* spores attached, respectively. * (n=24). ** Where the number 1 is equal to a J2's body length. [1] Nematode velocities were tested over a 6 minute period.

Reference

Vagelas IK; Pembroke B; Gravanis FT; Gowen SR and Kimber A 2006 The influence of *Pasteuria penetrans* on to second stage juveniles of *Meloidoyne* spp. movement, using digital motion image analysis. *XXVIII Symposium of the European Society of Nematologists* Blagoevgrad, Bulgaria, 5-9 June 2006.

Use of *Baculovirus* to control fall armyworm, *Spodoptera frugiperda*, in Brazil

F H Valicente, E Tuelher, R C Pena, R Andreazza, M R F Guimarães, C V Macedo
Embrapa Maize and Sorghum Research Center, 35701-970, Sete Lagoas, MG, Brazil
Email: valicent@cnpms.embrapa.br

A Gitz
Biocontrole Company, São Paulo, Brazil

J L C Wolff
Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil

Introduction

Fall armyworm, *Spodoptera frugiperda*, is the most important insect pest of corn in Brazil. Its control is essentially with chemical insecticides, but microbial pesticides may become a viable alternative. Among the insect pathogens, nucleopolyhedrovirus (NPV) is an option, and such baculoviruses can be very effective in the field control of fall armyworm. Advantages include no pollution of the environment or water and lack of adverse effects on natural enemies. However, two major problems have limited the large scale production of baculoviruses to control fall armyworm. First, fall armyworm is cannibalistic, so these insects have to be individualized, resulting in a lot of intensive lab work, a higher risk for contamination, and a high cost of the biopesticide. Secondly, baculoviruses that infect fall armyworm have the unique characteristic of disrupting the integument as soon as the larvae are dead. Cathepsin and chitinase genes are responsible for this. This makes production laborious and expensive, because larvae must be frozen and quickly harvested. Because a large quantity of the internal liquid containing the baculoviruses is lost, more larvae are needed to produce each dose sprayed. The objective of this work was to improve the large scale production of baculovirus biopesticides using one baculovirus isolate (isolate 6) that was found not to disrupt the integument.

Materials and methods

Bioassay 1: Baculovirus genomic DNA was isolated and the presence of chitinase and cathepsin genes in the genome of baculovirus isolate 6 was confirmed using PCR and specific primers. Sequencing showed no mutation in these two genes. We are now looking for some mutation along the genome.

Bioassay 2: The objective was to test larval weight as a parameter to quantify the dose needed per hectare (larval equivalent/ha). Leaves of maize and *Ricinus communis* (which is supposed to reduce cannibalism in fall armyworm) were used as substrate for infection of healthy larvae. Suspensions containing 1.35×10^6 parahedral inclusion bodies per ml (PIBs/ml) were sprayed on 5, 6 or 7 day old larvae on maize and *R. communis* leaves. Concentrations of 1.55×10^7 PIB/ml were used on 6 and 7 day old larvae. Results showed that all the correlations were positive and highly significant: at 1.35×10^6 PIB/ml (*R. communis*, n= 12, r= 0.987, T=19.63, $p<0.0001$ and maize: n= 12, r= 0.933, T=8.17, $p<0.0001$), and at 1.55×10^7 PIB/ml (*R. communis*, n= 16, r= 0.8456, T=5.93, $p<0.0001$ and maize, n= 16, r= 0.8591, T=6.28, $p<0.0001$). The final larval equivalents for *R. communis* and maize leaves were 13.9 and 13.7g of larvae/ha, respectively, at 1.35×10^6 PIB/ml. These results indicate that dead larval weight caused by baculovirus can be used as a reliable indicator for virus yield in a large scale baculovirus production.

Bioassay 3: The following parameters were determined in this assay: fall armyworm cannibalism, mortality caused by baculovirus, the amount of polyhedra per larvae, the weight and the larvae equivalent/ha necessary to achieve the recommended dose of 2.0×10^{11} PIB (before dilution in 250-300 liters of water/ha). Treatments were 6 and 7 days old larvae x period of feeding on leaves contaminated with baculovirus for 48 and 72 hours x two sources of food, leaves of *R. comunis* and maize (*Zea mays*). Isolate 6 (that does not disrupt the integument) was purified and used at 1.35×10^{7} PIB/ml plus Tween 20 and sprayed on the leaves of each substrate, with an overall use of 30 ml per treatment and per replicate. Seven hundred healthy larvae of *S. frugiperda* were transferred into each plastic recipient of 20 liters. The recipients were closed with a thin cloth and maintained under laboratory conditions (25.0 ± 1 ºC, 50.0 ± 10% r.h. and a light phase of 14h). All data were subjected to Three Way Analysis of Variance with the variables food substrate, larvae age and time of inoculation (larvae feeding on baculovirus). Cannibalism was significantly lower (<10%) in larvae fed for 48 hours on *R. communis* leaves than on maize, and after incubation for 72 hours mortality (>90%) of 6-day-old larvae was higher on maize leaves than on leaves of *R. communis*. The amount of polyhedra per dead larvae ($>2 \times 10^{9}$ PIB) was higher in seven-day-old larvae when fed on *R. comunis* leaves. Therefore, the larval equivalent/ha was lower for seven day old larvae (ranging from 100 to a 150 larvae/ha). However, there was no significant difference in the larvae weight needed to spray one 1 ha.

Bioassay 4: This assay evaluated the baculovirus virulence (infection of *S. frugiperda* larvae) after storage at room temperature. Ten batches of wettable powder formulation of baculovirus were tested, and each batch was composed of four replicates. Six day old larvae were used to evaluate mortality and tested in two different concentrations of each batch (10^{6} and 10^{7} PIB/ml) of isolate 6 (not disrupting the integument) and isolate 18 (batch 10-isolate disrupting the integument). Each replicate was composed of 24 larvae. After 48 hours, larvae were transferred to an artificial diet. Mortality was taken daily. Water and Tween 20 were used in the check treatment. Larval mortality was evaluated at time zero (T_0; immediately after formulation of the baculovirus) and the T_{1-3} months after the baculovirus was formulated. All data were subjected to a Two Way Analysis of Variance with the variables batch of baculovirus preparation and time. At a concentration of 10^{6} a decrease in mortality was observed in some batches. Shelf tests showed that for the concentration of 10^{6} PIB/mL the mortality of six day old larvae was lower than 90% at T_0, and for the concentration of 10^{7} PIB/mL, larval mortality was between 90 and 100% in most treatments. After three months of storage the mortality of batches containing 10^{7} PIB/mL remained unchanged, ranging from 90 to a 100%.

Conclusion

The new Brazilian isolate 6 of *S. frugiperda* that doesn't disrupt the integument was confirmed to harbour the cathepsin and chitinase genes. It proved very efficient for use in large scale baculovirus production. Using this isolate, the larval equivalent/ha could be lowered to 100 to 150 larvae/ha, equivalent to 10.75 and 13.86 g/ha, respectively. The virus preparation could be stored as a wettable powder for at least three months without a loss in mortality. Besides being efficient in killing fall armyworm, the virus was easy to harvest. This is the most important factor because dead larvae don't need to be frozen before being harvested, decreasing the need for space in freezers and the risk of contamination, and reducing the laboratory labour in larvae manipulation, all of which result in a decrease of the final cost of the biopesticide.

Do growth media and temperature affect the activity of *Beauveria bassiana* as a biological control agent ?

A Olleka, Sx Ren, Qb Hu
Laboratory of Biological Control, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China
Email: aolleka@yahoo.com

Introduction

Bemisia tabaci (Gennadius) (Homoptera: Aleyrodidae) is a worldwide pest of vegetable, ornamental, and field crops, causing both direct and indirect damage through feeding on plant sap. Direct feeding causes leaf chlorosis, a mottled appearance, and a reduction in plant vigor. Indirect damage is mainly through transmission of plant viruses, and to a lesser extent through promoting the development of sooty mould.

Biological control agents, such as pathogenic fungi, are an environmentally friendly alternative option for pest control. Their conidia attack the insect cuticle and germinate, entering into the hemocoel, and causing mortality through the depletion of hemolymph nutrients, or by producing toxic fungal metabolites. *Beauveria bassiana* (Balsamo) Vuillemin is one of the most widely studied and efficacious species of entomopathogenic fungi, with pathogenicity shown to about 200 different species of insects. Its development as a biological control agent has been of considerable interest, as problems with insecticide resistance and residues become limiting factors in crop production.

For successful epizootics, a high virulence of the fungi is important. Adequate virulence is dependent on the kind of culture medium and the generations of culture on laboratory media. The relative amounts of carbon and nitrogen available to the fungi determine whether they produce mainly conidia or mycelium. Entomopathogenic fungi must cope with different situations. They must be able to utilize different types of organic substrates as a source of energy and nutrients during culture in artificial media, and they must also be able to germinate on a susceptible host insect using the nutrients present on its cuticle.

Infection of insect pests by entomopathogenic fungi depends mainly on environmental conditions; indeed, sprayed conidia are exposed to a variety of environmental stresses, such as temperature, humidity, and photoperiod. Temperature is an abiotic limiting factor for conidial viability, germination and growth. In general, *B. bassiana* grows at a wide temperature range between 8 and 35°C. It is therefore important to match the thermal requirements of a prospective microbial control agent to the climatic conditions expected at the targeted environment and host.

The objectives of the present study were (1) to investigate the effect of different media on growth rate, sporulation and virulence of different isolates of *B. bassiana* and (2) to determine the thermal requirements for vegetative growth.

Materials and methods

Ten single-spore isolates derived from isolate Bb62 (originally isolated in Guangdong Forestry Institute from Pine moth) were used in this study. A suspension of 10^3 conidia/ml was used to inoculate Petri dishes (9 cm diameter), which were then incubated at 25°C, 60% relative humidity and a photoperiod of 16:8 hours (day/night).

Seventy-two hours post-inoculation young colonies originating form single spores were transferred to fresh plates and further cultured under the same conditions for 14 days.

(1) Petri dishes containing three different media, potato dextrose agar (PDA), Sabouraud dextrose yeast agar (SDAY) and Czapek Dox agar (Cz) were inoculated in the center with 5 µl of a conidial suspension (10^7 conidia/ml) of each isolate (4 plates per medium / isolate) and kept under the same conditions for 20 days. Colony diameters were measured every 2 days and used to calculate growth rates (mm/day). After 20 days of culture, Tween 80 was added to the plates. The plates were scraped with a spatula, and conidia in the resulting suspensions counted using a hemocytometer.

(2) The effect of temperature was evaluated by culturing isolate Bbs4 on SDAY at five different temperatures (17, 20, 23, 26 and 29°C with five replicates for each temperature). Growth rates and sporulation were determined as described above.

(3) To study the effect of media on virulence, suspensions containing 10^7 conidia/ml of each isolate were applied by spraying on second-instar nymphs of whitefly on the lower side of cabbage leaves. Application was done with a hand sprayer. Ten days after application, nymphs were inspected for the presence of fungal infections. Infected nymphs were easy to recognise by their reddish colour. Mortality was calculated using Abbot's formula. All results were subjected to one-way ANOVA followed by the Tukey test.

Results

(1) Only slight differences in growth rate were observed among the isolates on the same media, for instance, the mycelial growth on PDA ranged from 6.9 to 7.4 cm diameter (growth rate 3.5 to 3.7 mm/day), on SDAY from 5.4 to 6.4 mm diameter (growth rate 2.7 to 3.2 mm/day) and on Cz from 3.6 to 4.7 cm diameter (growth rate 1.8 to 2.4 mm/day). In contrast, significant differences were found for each isolate on different media. For example, the growth rate of isolate Bbs10 was 7.0, 5.4 and 4.2 mm on PDA, SDAY and Cz medium, respectively, indicating that the PDA media was the most suitable for vegetative growth followed by SDAY. Sporulation differed only slightly among the different isolates, whereas there was a significant effect of the culture media. For instance, Bbs9 produced 1.7, 0.9 and 0.1 $\times 10^5$ conidia/ml on SDAY, PDA and Cz medium, respectively. This demonstrates SDAY is the appropriate medium for conidia formation followed by PDA.

(2) Temperature had a significant effect on vegetative growth and sporulation of isolate Bbs4. The optimum temperature for growth and sporulation on the SDAY medium was 23 °C, whereas these parameters were lowest at 29 °C.

(3) Mortality of isolate Bbs7 on SDAY medium reached 79.3%. Conidia from PDA and Cz, however, caused a lower mortality, 73.5 and 49.6%, respectively. Performance of the other isolates also differed depending on the medium.

Discussion

The understanding of basic aspects of germination and growth of entomopathogenic fungi has greatly promoted the use of these organisms as biological control agents. Our results are in agreement with the findings of other scientists, demonstrating the effect of temperature and nutrients on growth and sporulation of entomopathogenic fungi. Knowledge of these factors is important for effective use of these fungi as agents for biological control of insect pests.

Elimination of Prunus necrotic ringspot and Arabis Mosaic Virus from rose plants by Stinging Nettle extract

F Rakhshandehroo, A Modarresi, H R Zamani Zadeh
Plant Pathology Department, College of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, PO Box-14515-775, Tehran, Iran
Email: farshadrakh@yahoo.com

Rose is an economically important crop of Iran and many other parts of the world and is among the most popular plants in Iran. Viruses that infect roses belong mainly to the genera *Ilarvirus* and *Nepovirus*. Viral diseases of roses do not usually kill infected plants but may reduce the plant vitality and quality of flowers (Horst, 1996). Rose mosaic is caused by a complex of several viruses including Prunus Necrotic Ringspot Virus (PNRSV) and Arabis Mosaic Virus (ArMV). They are not believed to occur naturally in roses however these viruses will survive in a rose bush and may be spread by grafting infected wood into other plants. It produces a range of symptoms according to the cultivars; although sometimes infected plants are asymptomatic (Moury *et al.*, 2000). Consequently, rose mosaic disease is a component of many phytosanitary certification programs. Medicinal plants have been traditionally used for different kinds of mammalian virus diseases but there have been few studies on the antiviral activity of plant extracts on plant viruses. Stinging nettle has been used for hundreds of years to treat rheumatism, eczema, arthritis, gout, and anaemia. Today, it is widely used to treat urinary and kidney problems. This study focused on the use of *Urtica dioica* (stinging nettle) for the production of mosaic virus-free rose plants by explant culture from infected parent material (*R. multiflora and R. chinensis*).

Urtica dioica plants were collected randomly from the highland regions in north part of Iran. Dried leaves and roots of nettle plants were extracted with water and 95% ethanol to obtain crude extracts. The dried sample was chopped into small parts with a blender. Water extraction of the active ingredient was performed according to the method of Agbafor (2004). Fresh leaves (200 g) of *U. dioica* were cut into pieces, homogenised and soaked in 400 ml of ethanol for 24 h before being filtered. The organic solvent was removed by rotary evaporation to obtain the crude active ingredient. Ethanolic extract was prepared by Soxhlet extraction of 20g of the powder with ethanol for about 10 hours according to the described method (Wang *et al.*, 2004). The solvent was removed under reduced pressure. For alcoholic extraction, dried leaves and roots of the plants were extracted with 95% ethanol at room temperature.

Plant extracts were used at three different concentrations of 10, 60 and 100 mg/ml (Plant extract/medium culture) for studying the antiviral activities. Each extract preparation was dispensed into Ms30 media containing the rose transplants. The control consisted of 10 transplants without plant extract. Each type of plant extract was tested twice with 20 rose plants infected with each virus. Detection of the viruses was carried out kits (Bioreba, Inc., South Bend, IN, USA). DAS-ELISA was conducted according to the general protocols (Moury *et al.*, 2000). Samples which were positive in reaction with any kind of antisera in ELISA were subjected to Dot-Blot Assay (DIBA) and RT-PCR using published protocols (Banttari & Goodwin, 1985; Verma *et al.*,(2002). The extracts of *Urtica dioica* exhibited virus inhibitory activity. ArMV was eliminated 90 percent of rose plants by using 60 or 100 mg/ml (aquatic nettle extracts) and 10 or 60 mg/ml (ethanolic extracts) respectively.

PNRSV was eliminated from 42 percent of tested plants by using 60 or 100 mg/ml (aquatic nettle extracts) and 10 or 60 mg/ml (ethanolic extracts) respectively.

The 100 mg/ml concentration of the ethanolic extract was toxic for roses and 10mg/ml concentration of aquatic extract ineffective. The inhibitory effects of plant extracts were also studied for the complex infection of ArMV and PNRSV. In the case of PNRSV, those samples that were negative for virus by serological methods were positive against the specific primer for the coat protein of PNRSV. Results of this study support the antiviral activity of *U. dioica* root and leaf extracts in the media containing the rose tissue cultures. According to the results, aquatic extracts of roots and leaves of nettle at the concentration of 100 mg/ml are more efficient in eliminating rose mosaic viruses than ethanolic extracts. PCR results indicate the presence of PNRSV within rose plants even after the treatment with aquatic nettle extracts but in such a concentration which is not detectable by serological methods. Initial screenings of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extractions and can be followed by various organic extraction methods.

Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. The exceptional water-soluble compounds, such as polysaccharides (e.g. starch) and polypeptides, including fabatin and various lectins, are commonly more effective as inhibitors of pathogen (usually virus) adsorption and would not be identified in the screening techniques commonly used.

Further studies of the effects of individual components alone and in combination will involve comparison with available viricides. Results of this study show that nettle extracts could be useful for production of virus-free rose explants. Although its use in mammalian medicine is widespread, such use of nettle extract in plant disease control is a novel application.

References

Agbafor KN (2004) The effect of aqueous and organic extracts of fresh leaves of *Baphia nitida* on tissue acetylcholinesterase in guinea pigs. *Journal of Science Technology* **10,** 1- 4.

Banttari EE and Goodwin, PH (1985) Detection of Potato Virus X and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (Dot-Elisa). *Plant Disease* **69**, 202-205.

Horst KR (1996) *Compendium of Rose Diseases*. American Phytopathological Society.

Moury B, Cardin, L, Onesto, JP, Candresse, T, Poupet, A (2000). Survey of Prunus necrotic ringspot virus in Rose and its Variability in Rose and Prunus. *Plant Disease* **91,** 84-91.

Verma N, Hallan, V., Zaidi, AA (2002). Detection of Prunus necrotic ringspot virus in begonia by RT-PCR. *Plant Pathology* 51, 800.

Wang W, Ben-Daniel, BH, Cohen, Y (2004). Control of plant disease by Extracts of Inula Viscosa. *Phytopathology* **94,** 1024-1047.

Fungitoxicity of *Inula helenium* extract against five pathogenic fungi

W Q Wang, W Y Zhang, X F Zhang, X Y Han, Z Q Ma, H X Li
Plant Protection Institute, Hebei Academy of Agricultural and Forestry Sciences, Baoding, 071000, China
Email: wenqiaow@163.com

Dry radix of *Inula helenium* (Compositae), a traditional herb in China, can be used for controlling human diseases caused by *Mycobacterium tuberculosis, Pseudomonas aeruginosa, Shigella* spp., *Staphylococcus aureus* because of its strong antimicrobial activity (Song *et al.* 2004). Isoalantolactone isolated from extract of *I. helenium* strongly inhibited some human pathogens e.g. *Aspergillus flavus, A. niger, Geotrichum candidum, Candida tropicalis* and *C. albicans* (Cantrell *et al.* 1999). The purpose of this study was to test the fungitoxicity to five pathogenic fungi of extracts from *I. helenium*, and to isolate and identify the antifungal compounds.

Dry radix of *I. helenium* was ground to a powder, two batches of powder were extracted in 99.9% methanol and 95% ethanol, respectively, for 3 h at 35°C at the ratio of 1:10 (powder/solvent, w/v), the extract was concentrated under reduced pressure to gain a paste which was dried at 60°C for 24 h in an oven. The dry paste of the methanol and ethanol extracts, with yield of 13.6% and 12.6%, respectively, were used to test the inhibitory activity against downy mildew (*Pseudoperonospora cubensis)* on detached leaves of cucumber and powdery mildew (*Sphaerotheca fuliginea)* on cucumber seedlings. The activity against mycelial growth of *Colletotrichum orbiculare*, *Botrytis cinerea* and *Fulvia fulva*, and conidial germination of *B. cinerea* was also tested. The results showed that, compared with the methanol extract, the ethanol extract was much more inhibitory against *P. cubensis* and *S. fuliginea* (Table 1) and against mycelial growth of *C. orbiculare*, *B. cinerea* and *F. fulva*, and conidial germination of *B. cinerea* (Tables 2 and 3).

Spraying cucumber seedlings with 5 mg/ml methanol extract and spraying detached cucumber leaves with 50mg/ml of methanol extract gave control efficacy of 69.2% against powdery mildew and 81.5% against downy mildew. The ethanol extract giving 99.9% against powdery mildew and 96.3% with downy mildew at the same concentrations. Mycelial growth of *C. orbiculare*, *B. cinerea* and *F. fulva* was inhibited by 50mg/ml of methanol extract and ethanol extract coated evenly on PDA plates by 32.0%, 43.2% and 34.6% for methanol extract, and 57.4%, 91.6% and 79.0% for ethanol extract.

An aqueous solution of ethanol extract was partitioned with either petroleum ether, chloroform or acetic ether (1:1). *B. cinerea* mycelial growth on PDA was strongly inhibited by petroleum ether fraction and chloroform fraction of ethanol extract (EC_{50} 1.71mg/mL and 1.30mg/mL), mycelial growth of *C. orbiculare*, *B. cinerea* and *F. fulva* was strongly inhibited by the petroleum ether fraction (EC_{50} 1.73mg/mL and 7.43 mg/mL), but the aqueous phase fraction exhibited no inhibitory activity (Table 3).

A crystal was gained through recrystallization of the petroleum ether fraction of the ethanol extract, and identified as isoalantolactone through the external standard of HPLC and ^{13}C-NMR. 0.01g/ml of isoalantolactone inhibited mycelial growth of *B. cinerea* by 66.73% and *F. fulva* by 58.11% on PDA.

In conclusion, an ethanol extract of *I. helenium* was more efficient at inhibiting five pathogenic fungi on cucumber and tomato than the methanol extract. Antifungal compounds may exist mainly in fractions of solvents (i.e. petroleum ether) with lower polarity. Isoalantolactone, a lipophytic compound with high activity against the five pathogenic fungi was isolated from the ethanol extract of *I. helenium*. Radix of *I. helenium* may be used as a resource of plant-derived fungicides.

Table 1. Inhibitory effect of extracts from *I. helenium* against *P. cubensis* and *S. fuliginea*

Fungi	Extract type	EC_{50}(mg/mL)
P. cubensis	methanol	8.6[b]
	ethanol	0.7[a]
S. fuliginea	methanol	2.1[b]
	ethanol	0.18[a]

Values in columns followed by different letters are significantly different (P=0.05) according to Fisher's protected least significant difference test.

Table 2. Effect of extracts from *I. helenium* on mycelial growth and conidial germination of pathogenic fungi

Fungi	Extract type	Development stage	EC_{50}(mg/mL)
C. orbiculare	methanol	mycelial growth	222.6[b]
	ethanol	mycelial growth	36.1[a]
F. fulva	methanol	mycelial growth	84.7[b]
	ethanol	mycelial growth	13.8[a]
B. cinerea	methanol	mycelial growth	162.2[b]
	ethanol	mycelial growth	15.2[a]
B. cinerea	methanol	conidial germination	4.40[a]
	ethanol	conidial germination	5.17[b]

Letters indicate significance as in Table 1

Table 3. Effect of various fractions from ethanol extract of *I. helenium* on mycelial growth of three pathogenic fungi

Type of fraction	EC_{50}(mg/mL)		
	B. cinerea	*F. fulva*	*C. orbiculare*
Petroleum ether	1.71[a]	1.73[a]	7.43[a]
$CHCl_3$	1.30[a]	11.54[b]	24.48[b]
EtOAc	12.94[b]	12.29[b]	97.03[c]
Water	6376[c]	2.9×10^{37}[c]	2138[d]

Letters indicate significance as in Table 1

References

Song SQ; Zhou LG; Hao XJ; Li JQ (2004). Resources of plants with antimicrobial activity in Xinjiang. *Acta Botanica Boreali-Occidentalia Sinica* **24 (2)**, 259-266.

Cantrell CL; Abate L; Franzblau SG; Franzblau SG; Quijano L; Fischer NH (1999). Antimycobacterial eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa*. *Planta Medica* **24 (2)**,351-355.

New *Bacillus* spp. strains isolated from natural sources or genetically modified with increased antimicrobial activities

C M Voaides, C P Cornea, M Ciuca, R Tezel, G Popa, N Babeanu
Faculty of Biotechnology, UASVM Bucharest, 59 Bd. Marasti, Bucharest, Romania
Email: catalinavoaides@gmail.com

The struggle against fungal and bacterial plant pathogens is an actual problem. Damage results not only from quantitative losses, but also from impairment of the quality of the produce, e.g. due to mycotoxins formed during pathogenesis. There is an increasing interest among biologists in using beneficial micro-organisms as a solution to the overuse of potentially harmful pesticides. The interest throughout the world for biocontrol of plant pathogens is promoted by significant restrictions in the use of chemical products. Nature has its own remedies for plant diseases. An example in this respect is represented by different species of *Bacillus*, isolated in our laboratory and in others, which produce metabolites with antifungal activity that are regarded as 'friendly' to the environment (Cornea *et al.*, 2003; Mateescu *et al.*, 2005). Accordingly, the aim of our research was the isolation and selection of new *Bacillus* spp. strains with antimicrobial activities and their identification, as well as the improvement of their antimicrobial activity by mutagenesis and genetic transformation.

Bacteria with antimicrobial activity were isolated from soil. Appropriate serial dilutions from soil suspensions in sterile H_2O were plated on King B plates and the plates were incubated at 30°C for 48 h. Single colonies were isolated and screened for antifungal activity by using the Petri plate assay described by Manka (1992). Activity against the plant pathogenic bacteria *Pseudomonas syringae* and *Erwinia amylovora* was evaluated in a Petri plate assay. The pathogens and the *Bacillus* strains to be tested were placed 2 cm apart on agar medium in Petri dishes. The inhibitory effect of the *Bacillus* spp. strains was determined by measuring the colony diameter of the bacterial pathogens at five days after inoculation of the plates. In some cases a precipitation line between the *Bacillus* colony and the pathogen was noticed. Although the significance of this observation is not understood, it could be explained as an interaction, typically for lectins. Three of the newly isolated bacilli had a wide spectrum of activity and were active against various plant pathogenic fungi (*Botrytis cinerea, Alternaria tenuis, Fusarium* spp., and others) and/or the plant pathogenic bacteria employed (*P. syringae, E. amylovora*). For further studies the three pathogens *A. tenuis, P. syringae* and *E. amylovora* were selected based on their economic importance for the Romanian agriculture. In order to identify the new isolates, the BIOLOG system as well as a molecular technique (RAPD) was employed. Using these methods, two of the isolates were identified as *Bacillus licheniformis* (designated as strains B2R and Bw) and one as *B. subtilis* (strain BsC).

We also started experiments to explain the mechanisms of action involved in the antimicrobial activity of the three novel strains. In these preliminary studies we observed that the antimicrobial activity was caused by inhibitory compounds resembling in structure and activity antibiotics of three groups of lipopeptides (Phae *et al*, 1990): the surfactin group, the plipastatin-fengycin group and the iturin group (Tsuge, 2001). Extraction of iturins from the culture medium was performed according Phae *et al.* (1991).

The antagonistic properties of strains B2R, Bw and BsC may also be explained by their capacity to produce HCN. Although this ability is representative for *Pseudomonas* spp., other *Bacillus* strains from our collection have shown an increased capacity to produce HCN. Production of HCN was evaluated according to the method of Lork. The results obtained showed that the cyanogenic capacity of the three *Bacillus* spp. strains was visibly lower than that of pseudomonads. This observation suggested that more mechanisms are involved in the antimicrobial activity of strains B2R, Bw and BsC. The three novel *Bacillus* spp. strains were compared with other bacterial strains known as biocontrol agents from the collection of the Laboratory for Genetics and Genetic Engineering of the Faculty of Biotechnology. The production of indol-acetic-acid (IAA) was determined according to the method of Bano (2003). IAA-positive strains developed a pink colour that was measured spectrophotometrically (absorbance at 530 nm). The IAA concentration in the culture broth was quantified by linear regression analysis using a calibration curve of pure IAA. The results showed that B2R, Bw and BsC are able to produce IAA, but in small quantity. Whether this IAA production can lead to plant growth promotion will be analysed in further in vivo studies.

Obtaining large quantities of biologically active substances is often hampered by their low level of biosynthesis. Until recently, improvement of the inhibitory abilities of bacilli was restricted to techniques involving mutagenesis. The development of molecular techniques now opens the perspective of obtaining recombinant bacterial strains capable of synthesizing elevated quantities of the desired product. In order to improve the antimicrobial and potential plant growth promotion activity of B2R, N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis was performed, and the strain was transformed by electroporation with pLC1 plasmid. Mutants and transformants selected were tested against phytopathogenic fungi and/or bacteria.

Although still at an early stage, the results on the use and improvement of strains B2R, Bw and BsC as biocontrol agents of various plant pathogens obtained so far are very promising.

References

Bano, N; Musarrat J (2003). Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. Current Microbiology **46**, 324-8.

Cornea, CP; Mateescu, R; Grebenişan, I; Vamanu, E; Câmpeanu, G (2003). Isolation and characterization of new *Bacillus* spp. strains–useful as biocontrol agents of plant pathogens. Roumanian Biotechnology Letters **1**, 1115 – 1122.

Manka, K; Manka, M; (1992). A new method for evaluating interaction between soil inhabiting fungi and plant pathogens, Bulletin OILB/SROP, XV, 1.

Mateescu, R; Cornea, CP; Grebenişan, I; Câmpeanu, G (2005). Mechanism involved in the reduction of a plant disease caused by *Alternaria tenuis* and plant growth promotion using *Bacillus spp.* strains. Roumanian Biotechnology Letters **10**, 2403-2408.

Phae, CG; Shoda, M; Kubota, H; (1990). Supressive effect of *Bacillus subtilis* and its products on phytopathogenic microorganisms. Journal of Fermentative Bioengineering **69**, 1-7.

Phae, CG; Shoda, M; (1991). Investigation of optimal conditions for foam separation of iturin, an antifungal peptide produced by *Bacillus subtilis*. Journal of Fermentative Bioengineering **71**, 118-121.

Tsuge, K; Akiyama, T; Shoda, M (2001). Cloning, sequencing and characterization of the iturin A operon. Journal of Bacteriology **183**, 6265-6273.

The association of differentially-expressed proteins with maize resistance to *Curvularia lunata* (Wakker) Boed in China

J Chen, X Huang, L Liu, Y S Xu, Y Zhai
School of Agriculture & Biology, Shanghai Jiaotong University, Dongchuan Road 800, China
Email: jiechen59@sjtu.edu.cn

Curvularia leaf spot of maize, caused by *Curvularia lunata*, is widely distributed throughout the world. A proteomic approach was applied to locate resistance genes, by analysis of expressed proteins from different inbred maize lines. All samples were taken from 4 inbred lines inoculated with the pathogen in the presence or absence of *Trichoderma atroviride* in rhizosphere soil. High-resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry were employed to identify proteins that are differentially expressed in response to fungal infection in maize leaf. From over 300 spots, thirty eight spots were selected and subjected to tryptic digestion followed by identification using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS/MS) and nanospray ion-trap tandem mass spectrometry.

The differentially-expressed proteins were split into four groups according to known functions. The first group, mainly involved in photosynthesis, were Rubsico and chlorophyll a/b-binding protein. The second group, associated with respiration metabolism, included PEPase, P-pyruvate carboxylase, fructose 1, 6-bisphosphate aldolase and glyceraldehyde 3-phosphate dehydrogenase. The third group, dedicated to bioenergy metabolism, included ATP synthase and ATPase. The fourth group, with a series of stress-mediated proteins, included germin-like protein, 22kDa drought-inducible protein, translation initiation factor 5A, glutathione peroxidase, ascorbate peroxidase, ABA-and ripening-inducible-like protein and oxygen evolving enhancer protein 1 (OE1). Some unknown proteins might also participate the regulation of host defence response.

Proteins in the fourth group seemed to be most associated with the maize leaf defence response to *C. lunata* infection, in which germin-like protein (GLP), putative glutathione peroxidase (GPX), 22kDa drought-inducible protein and translation initiation factor 5A (eIF-5A) were only shared among resistant inbred lines (Luyuan 92 and 78599-1), this is similar to the findings of Chen *et al.*, (2004). GLPs have been associated with activity of superoxide dismutase (SOD) or oxalate oxidase (OXOX) (Bernier & Berna, 2001). These enzymes function as scavengers to remove the accumulation of active oxygen radicals during pathogen invasion. Therefore, putative glutathione peroxidase (GPX) does not only mediate host defence response but may play a role in a coordinated way to reinforce maize defence response against *C. lunata* infection.

The 22kDa drought-inducible protein appears to be a component of the anti-oxidation system in the host plant and also a protein involved in the host response to pathogen infection, particularly when *Trichoderma atroviride* is present in the corn rhizosphere. Proteins related to host drought tolerance contribute to host resistance to fungal pathogen infection (Chen *et al.*, 2004). Disease susceptibility has increased in Northern China following frequent drought period over the past decade.

P6D-19

Eukaryotic translation initiation factor 5A (eIF-5A) is one of the factors necessary for the initiation of eukaryotic cellular protein biosynthesis and mRNA selective transport across the membrane in plant developmental and environmental response. In our studies, the expression levels of eIF-5A (spots 2, 31) were increased specifically in resistant inbred lines upon pathogen infection.

Apart from specifically induced proteins in resistant inbred lines, some proteins were common in both resistant and susceptible inbred lines. However, some were up-regulated in resistant inbred lines, such as OEE1, which eliminates accumulated reactive oxygen species (ROS) in maize leaf. ABA- and ripening-inducible-like proteins were up-regulated in resistant inbred line78599-1, and down-regulated in susceptible inbred line Huangzao 4. ABA is significant in the regulation of plant stomata against drought stress and host defence response. The coordinated role of ABA- and drought- inducible proteins should contribute to greater host tolerance than a single component. Similarly ascorbate peroxidase (APX) was found to be common to both inbred lines, but its expression was much higher in resistant inbred lines. APX is an antioxidant, similar to GPX, which is involved in the removal of active oxygen radicals. GLX1 as a member of the glyoxalase system (Bernier & Berna, 2001), was specifically induced by *Trichoderma atroviride* in the presence of the pathogen, which made glutathione (GSH)-based detoxification more effective.

The proteins in the fourth group are induced in resistant inbred lines or in the presence of *Trichoderma atroviride* in a coordinated manner to remove ROS and increase host tolerance to drought stress, which subsequently reinforces maize leaf defence response against *Curvularia lunata* infection. Therefore it would be reasonable to view them as genetic markers of host resistance. The other three groups of proteins support, in an indirect way, the regulation of a complex defence mechanism.

Acknowledgement

We thank Professor Gary C Harman, Cornell University, USA for discussions. Grants provided by: the National Natural Science Foundation (30471057); the National Basic Research Program of China (2006CB101901) and the national high technology research and development program of China (2006AA10A211).

References

Chen, ZY Brown, RL and Cleveland, T (2004). Evidence for an association in corn between stress tolerance and resistance to *Aspergillus flavus* infection and aflatoxin contamination. African Journal of Biotechnology 3: 693-699.

Bernier, F and Berna, A (2001). Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? Plant Physiology Brioche. 39: 545-554.

Effect of biocontrol on Fusarium wilt of cucumber and the influence of *Trichoderma atroviride* strain T23 on cucumber defence enzyme activities

J Chen
Shanghai Jiaotong University, Shanghai 201101, China
Email: jiechen59@sjtu.edu.cn

J Zhuang
Shenyang Agricultural University, Shenyang 110161, China

In Northern China, cucumber Fusarium wilt (caused by *Fusarium oxysporum*) often leads to serious reductions in vegetable production. Numerous studies on the biocontrol of the disease with *Trichoderma* and other biocontrol agents have been performed. However, until now, there has been little research aimed at comparison of the relative contributions of conidia and chlamydospores of *T. atroviride* T23 to induced resistance against cucumber fusarium wilt. The objective of the study described here is to detect dynamic changes of several important defence response–related enzymes in cucumber roots, induced by either Trichoderma conidia or chlamydospores, or both. This work will allow us to further understand the biocontrol mechanism of *Trichoderma* against fusarium wilt *in planta*.

The defence response enzymes in cucumber root in these experiments were analysed using a bioassay approach. T23 conidia and chlamydospores were prepared as spore suspensions (at concentrations of 5×10^7 cfu/ml for conidia, and 2×10^6 cfu/ml for chlamydopores). The suspension was poured into rhizosphere soil of cucumber at the four leaf stage, two days prior to pathogen inoculation. Disease assessment and root sampling were conducted at 1d, 3d, 5d, 7d, 10d, 13d and 16d after T23 inoculation.

After the inoculation with T23 conidia and chlamydospores, the fusarium disease index in control plants (inoculated with the pathogen only) was 33.69, whereas the disease index in plants treated with T23 chlamydospores, prior to inoculation with the pathogen, was 10.28 and the disease index in those treated with conidial suspensions, prior to inoculation with pathogen, was 3.12.

Spectrophotometric analysis showed significant increases in the activities of peroxidase (POD), phenylanine ammonia lyase (PAL), polyphenoloxidase (PPO) and catalase (CAT) in cucumber plants were observed, regardless of treatment with conidia or chlamydospores. However the highest peak value was up to 2.75 (PAL), 2.49 (POD), 2.42 (PPO) and 15.84 (CAT) times higher over control treatment when T23 chlamydospore were inoculated, as compared with conidia treatment. Chlamydospores usually resulted in activity peak of PAL coming later but higher in activity intensity, hence PAL production appeared to be promoted by chlamydospores more than conidia spores.

There were three peaks of POD activity at five, 10 and 16 days after inoculation with *Trichoderma* chlamydospores and pathogen suspension whereas, in the control treatment, no peak of POD activity was visible and production remained at low level. PPO activity peaked at three days, and then gradually declined to a stable level at five days after inoculation with *Trichoderma* conidia and pathogen. However, there was a fast growth of enzyme activity at five days, eventually up to maximum peak (589.93 Umg^{-1}protein) after 13 days, 2.41 times greater than control, but no PPO peak was detected in the control.

CAT activity increased significantly and peaked and stabilised at three days after inoculation with either conidia or chlamydospores in cucumber roots, which was 4.14 and 4.44 times higher than the control. CAT activity started to increase at seven days and peaked at 10 days after inoculation with Trichoderma chlamydospores and pathogen suspension, 10.45times greater than that in the control. Another peak (301.06 Umg-1protein) emerged at 16 day after inoculation in the same treatment, which was 15.84 times than the control treatment.

Taken together, the presence of conidia resulted in high enzyme activity earlier than when chlamydospores were present, but the latter usually induced a higher enzyme activity peak than the former. Therefore, chlamydospores seemed to be more effective in the induced defence response of cucumber against Fusarium attack.

This data provides strong evidence that lignin and phytoalexin and other biochemical substances, whose synthesis was usually regulated by the enzymes studied, were probably involved in the induced host defence response against Fusarium attack (Howell & Hanson, 2000; Nzojiyobirijean & Xu, 2003; Yedidia & Benhamou, 1999). Based on previous research, PAL activity in host plants often emerges very early during pathogen attack, as does POD activity, which is involved in the synthesis of lipid and oxidization of aromatic and phenol substances which then are required for lignin synthesis. PPO enables phenol to be oxidized into quinones which has been verfied toxic to pathogens. CAT is a hydroxyl radical scavenger, which reduces over-acumulation of active oxygen radicals. Thus the enzymes are likely to have been involved in the prevention of plant senescence. In conclusion, all those enzymes studied play a significant role in host defence response to the disease infection.

Acknowledgement
We thank the National Ministry of Science and Technology, China for their support in providing a High Technology Development Project grant (2006AA10A211) and the Agricultural Ministry, China for providing a project grant (2006--G54(A) provided.

References

Howell, CR; Hanson, LE; Stipanovic, RE and Puckhaber, LS (2000) Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology **90**, 248-252.

Ying, S; Nzojiyobiri, JB: Tong, X and Ming, SF (2003) Resistance induced by *Trichoderma harzianum* NF9 against *Magnaporthe grisea* and *Xathomonas oryzae* pv. *oryzae* in Rice. Chinese Journal of Biological Control **19(3)**, 111-114.

Yedidia, I; Benhamou, N and Chet, I (1999) Induction of defence responses in cucumber plants (Cucumis sativus L.) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microbiol. **65(3)**,1061-1070.

Biopriming of sunflower seeds – a potential tool for increasing the efficacy of biological seed treatment for the management of Alternaria blight of sunflower

M S L Rao
Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Dharwad, 580005, India
Email: mslrao@gmail.com

S Kulkarni, S Lingaraju, H L Nadaf
Department of Plant Pathology, University of Agricultural Sciences, Dharwad - 5, India

Seed treatments provide economical and relatively nonpolluting delivery systems for protective materials compared to other field application systems. Bioprotectants applied to seeds may not only protect seeds but also may colonize and protect roots and increase the plant growth. However, biological agents have tended to be somewhat less effective and more variable than chemical seed treatments. Thus, seed treatment systems that will enhance the efficacy of biological agents are needed and 'biopriming' is one such attempt being made in this direction. Seed treatment with biocontrol agents along with priming agents may serve as an important means of managing many of the soil and seed-borne diseases, the process often known as biopriming (Taylor & Harman, 1990). Hence, the present investigation was undertaken to evaluate the role of biopriming in enhancing the efficacy of biological seed treatment for the management of Alternaria blight of sunflower - a potentially destructive disease in many parts of the sunflower growing countries.

For biopriming, one hundred grams of infected sunflower seeds (Hybrid-KBSH-44) were treated with the bacterial biocontrol agent *Pseudomonas fluorescens* at 0.8% concentration (Commercial formulation- Biocure from T Stanes India Ltd.) with priming agents like vermiculite and jelly (water absorbent polymer) in the proportion of 3:1 (3 parts of vermiculite or jelly and 1 part of seed) and mixed thoroughly to give uniform coating. Seeds were also treated with carbendazim+ iprodione (Quintal) at 0.3 per cent concentration. A field trial experiment was laid out in a randomized block design with three replications. The treatments were used in combination with a commonly used foliar spray of 0.1 per cent hexaconazole at 40 days after sowing. To see the effect of biopriming of sunflower seeds with *P. fluorescens* in different priming agents, on the activity of defense related enzymes like peroxidase, poly phenol oxidase and catalase, isozyme studies were undertaken as per the standard procedures. The isozyme analysis of peroxidase, polyphenol oxidase and catalase was done by using vertical slab Poly Acrylamide Gel Electrophoresis (PAGE) technique.

Results of the *in vitro* evaluation of integrated seed treatment options revealed the increased efficacy of bioprimed seeds with *P. fluorescens* (at 0.8%) in jelly compared to direct seed coating with *P. fluorescens* indicating the significance of priming. In the investigation on field evaluation of integrated seed treatment options, *P. fluorescens* performed better, in terms of yield and benefit-cost (B:C) ratio, when jelly was used as the priming agent along with foliar spray of hexaconazole compared to direct seed treatment with *P. fluorescens*, seed treatment of *P. fluorescens* with vermiculite, seed treatment with carbendazim+ iprodione (Table 1). Isozyme studies indicated that bioprimed seeds exhibited enhanced activity of defence related enzymes like peroxidase, polyphenol oxidase and catalase indicating the probable mode of action of these bioprimed seeds in affording protection against seed and seedling infection of Alternaria.

Table 1: Field evaluation of integrated seed treatment options with priming agents for the management of Alternaria leaf blight of sunflower (Hybrid: KBSH-44)

Treatment†	Per cent Disease Index at 45 DAS	60 DAS	75 DAS	Head diameter (cm)	Test weight (g)	Yield (q/ha)	B: C ratio
Q.H.	24.91 (29.88)	38.97 (38.62)	47.60 (43.62)	23.00	4.03	15.08	8.98
PF+H	40.7 (39.63)	49.58 (44.75)	56.98 (49.02)	19.33	3.8	13.33	4.41
PFVH	36.26 (37.00)	43.66 (41.36)	48.59 (44.19)	20	5.01	15.27	6.24
PFJelly H	29.60 (32.96)	39.22 (38.76)	45.63 (42.43)	23.5	5.45	15.92	10.53
NH	44.15 (41.64)	57.47 (49.51)	65.86 (54.25)	16.33	3.62	14.99	8.76
NQH	40.70 (39.62)	50.56 (45.21)	67.34 (55.14)	18.86	4.43	15.27	9.38
NPFH	44.64 (41.91)	59.94 (50.73)	64.97 (54.33)	18.60	4.10	15.04	8.85
H alone	44.76 (41.95)	62.00 (52.06)	64.71 (53.55)	22.83	3.45	13.36	4.52
Control	61.17 (51.58)	67.83 (55.79)	76.22 (60.80)	13.33	3.18	11.66	
S.Em ±	0.64	0.67	0.62	0.34	0.09	0.15	
CD @ 5%	2.45	2.57	1.68	1.31	0.37	0.59	

∗ Figures in parentheses indicate arcsine-transformed values

†Key

Q.H.	:	Carbendazim + Iprodione (Quintal) seed treatment (0.3%)+ Hexaconazole spray (0.1%) at 40 Days After Sowing (DAS)
PF+H	:	Direct seed treatment - *Pseudomonas fluorescens* (0.8%)+ Hexaconazole spray (0.1%)
PFVH	:	*P.fluorescens*(0.8%) + Vermiculite + Hexaconazole spray (0.1%)
PFJelly H	:	*P.fluorescens*(0.8%) + Jelly + Hexaconazole spray (0.1%)
NH	:	Seed treatment with Neem leaf extract (10%) + Hexaconazole spray (0.1%)
NQH	:	Neem leaf extract (10%) + Quintal + Hexaconazole spray (0.1%)
NPFH	:	Neem leaf extract + *P.fluorescens* + Hexaconazole spray (0.1%)
H alone	:	Hexaconazole spray alone
Control	:	Untreated

Reference

Taylor, AG & Harman, G E (1990). Concepts and technologies of selected seed treatments. Annual Review of Phytopathology, **28,** 321-339.

Screening of pimaricin and pimaricin-like metabolites of *Streptomyces natalensis* for control of phytopathogenic fungi

D Stephan, E Koch
Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Heinrichstr. 243, D-64287 Darmstadt, Germany
Email: D.Stephan@bba.de

Pimaricin, a polyene macrolide antibiotic, is produced by *Streptomyces natalensis* and *S. chattanoogensis*. It is a well described fungicide that binds to steroles, thereby changing the permeability of the cell membrane. Pimaricin has been used as a pharmaceutical for treatment of fungal infections or to disinfect cheese rind. It is not known whether this light and oxidation sensitive molecule can be used as an agricultural fungicide. We investigated the effect of commercial pimaricin and of pimaricin-like metabolites produced by *Streptomyces natalensis* on phytopathogenic fungi *in vitro* and *ad planta*.

Material and methods

For determination of the effective concentrations (EC 50 and EC 90) needed to inhibit various fungi, Pimaricin was added to autoclaved Czapek-Dox agar (for *Botrytis cinerea, Drechslera tritici repentis, D. teres, , Fusarium. culmorum, F. nivale, Gaeumannomyces graminis, Mortierella alpina, Pythium ultimum, Rhizoctonia solani, Sclerotium cepivorum, Venturia inaequalis*) or rye-sitosterol agar (for *Phytophthora infestans*) at final concentrations of 0.1, 1, 10 and 100 ppm. The plates were centrally inoculated with 5 mm discs of two week old fungal cultures and incubated at 20 ± 1 °C (15 °C for *P. infestans*) in darkness. Radial mycelial growth was measured when at least 75% of the control plates (not amended with pimaricin) were covered by the fungus. Five Petri dishes were inoculated per treatment. Using the measured inhibition, EC 50 and EC 90 values were calculated by non-linear regression analysis with the equation $f=a*x^b/(c^b+x^b)$ (Hill, with the three parameters a, b, c) using Sigmaplot Vers. 8.0. For the *ad planta* experiments, a *Streptomyces natalensis* mutant was provided by ASA Spezialenzyme, Wolfenbüttel, Germany. This mutant had previously been selected for high production of pimaricin-like metabolites. It was cultured in Erlenmeyer flasks in a liquid medium containing 0.4% glucose, 0.4%yeast extract, 1.0% malt extract and 0.2% $CaCO_2$ for seven days at 30°C and 180 rpm on a rotary shaker. After fermentation, the culture was centrifuged. The pimaricin concentration in the supernatant was determined following the description of Fletouris *et al.* (1995). Using an application rate of 100 ppm, the efficacy of pimaricin and the pimaricin-like metabolites was evaluated in standardised bioassays with different pathosystems. Depending on the pathosystem, the commercial products Atempo, Saprol, Diskus, Euparen, Arena C or Abavit were included. Depending on the test, the parameters evaluated were Area under the Disease Progress Curve (AUDPC), % leaf area affected or % infected plants. All experiments were repeated three times.

Results

The results of the *in vitro* tests (Table 1) show that pimaricin affected mycelial growth of a wide range of phytopathogenic fungi, with the exception of the two tested oomycetes, *Phytophthora infestans* and *Pythium ultimum*. In contrast to the situation *in vitro*, pimaricin was effective against *P. infestans ad planta* (Table 2). This can be possibly explained by inhibition of zoospore release by pimaricin. Only limited control was achieved in case of *S. fuliginea, V. inaequalis, T. caries* and *D. teres*. However, control of *B. cinerea* by pimaricin was comparable to the level of control provided by the chemical standard.

Table 1: Inhibitory activity of pimaricin on the mycelial growth of different phytopathogenic fungi on agar medium

Species	Regression function $f=a*x^b/(c^b+x^b)$ with the parameters			R[1]	Effective conc. (ppm)	
	a	b	c		EC 50	EC 90
P. infestans	35.64	4.35	72.8	0.74	>100	>100
P. ultimum	-	-	-	-	>100	>100
S. cepivorum	100.0	4.71	0.75	1.00	0.8	1.2
D. tritici repentis	97.79	0.96	0.81	0.97	0.9	10.4
G. graminis	100.5	1.54	2.88	1.00	2.9	11.7
F. culmorum	100.8	1.47	3.18	1.00	1.4	13.4
F. nivale	93.10	3.55	3.40	1.00	3.5	8.6
V. inaequalis	108.4	0.79	4.32	1.00	3.6	32.0
R. solani	101.1	1.35	3.01	0.98	23.9	72.3
D. teres	91.58	5.14	6.89	0.93	7.1	15.1
M. alpina	101.2	1.35	3.01	0.98	8.6	11.3
B. cinerea	97.81	3.06	4.20	1.00	4.3	9.3

1= Regression coefficient

Table 2: Effect of commercial pimaricin and of pimaricin-like metabolites produced by *Streptomyces natalensis* on different fungal pathogens in plant bioassays

Pathosystem	treatment / reading value			
	pimaricin	pimaricin-like metabolite	chemical standard	untreated
P. infestans on detached potato leaves *	56.6 *b*	91.0 *b*	55.6 *b*	280 *a*
P. infestans on potted potato plants **	36.9 *a*	42.2 *a*	28.1 *a*	72.7 *b*
S. fuliginea on cucumber **	84.3 *a*	88.6 *a*	11.2 *b*	87.0 *a*
V. inaequalis on apple seedlings **	88.9 *a*	77.8 *a*	0.00 *b*	96.3 *a*
B. cinerea on *Vicia faba* leaves **	17.4 *b*	13.4 *b*	5.20 *b*	96.5 *a*
T. caries on wheat ***	72.0 *b*	73.6 *b*	0.00 *c*	89.5 *a*
D. teres on barley ***	20.9 *b*	27.0 *a*	3.34 *c*	29.07 *a*

*= Area under disease progress curve, **=% affected leaf area, ***=% infected plants
Means followed by the same letter are not significantly different following SNK ($p < 0.05$).

The results indicate that *in vitro* assays do not necessarily represent the efficacy of pimaricin *ad planta*. Only in case of *B. cinerea* did the low EC-values determined *in vitro* correlate with good disease control. In most pathosystems the pimaricin-like metabolites were similarly as effective as the commercial pimaricin. With both, good control of *B. cinerea* was achieved at 10 ppm. Additional experiments showed that the pimaricin-like metabolites can easily be formulated by freeze-drying without loss of efficacy.

Reference

D J Fletouris; N A Botsogkou; A J Mantis (1995). Rapid spectrophotometric method for analyzing natamycin in cheese and cheese Rind, *Journal of AOAC International* **78**, 1024-1029

The extraction, purification and identification of the antifungal substance produced by *Streptomyces lydicus* A02

W C Liu, J Y Qiu, T Liu, C G Lu
Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China
Email: qiujiyan@yahoo.com.cn

Q Sui, X M Liu
Department of Plant Protection, Northeast Agricultural University, Harbin 150030, China

Introduction

According to the literature, 80% of the bioactive substances used as agricultural antibiotics are produced by species of *Streptomyces* from soil. So exploiting actinomycetes from soil is an important approach for developing agricultural biocontrol agents, especially fungicides. *Streptomyces lydicus* A02 was isolated from a forest soil in Beijing suburb. In a previous study, the strain presented a stable and strong antagonistic activity against many plant pathogenic fungi, such as *Botrytis cinerea*, *Alternaria solani*, *Fusarium graminearum*, *Rhizoctonia cerealis*, and others. In potted plant trials performed in the greenhouse, high control efficiencies towards vegetable grey molds (up to about 90%) were obtained with the sterile filtrate from fermented broth of strain A02. It is obvious that the strain is able to produce efficient antifungal substances. In this research, the active metabolic product was extracted, purified and identified.

Materials and methods

S. lydicus A02 was provided by Microbial Engineering Laboratory, Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences.

The culture and fermentation of strain A02 was carried out according to the method of Pan *et al.* (2005). The fermented broth was mixed with 100 % ethanol (1:3, v/v), placed at 4 C for 2 h, then stirred up again and centrifuged at 5,000g for 20 min. The supernatant was concentrated using a rotary evaporator at 45 °C. The concentrate was absorbed into macroporous resin X-5 (Chemical factory of Nankai University, China) and eluted with deionised water, 30% methanol, and 70% ethanol in proper order. The eluent was collected into tubes in portions of 5 ml. After being concentrated with a SpeedVac centrifugation concentrator (Thermo SPD1010) and bio-assayed using the agar diffusion method with *Botrytis cinerea* as the indicator, the active fractions were separated again by using a 200 mesh silica gel chromatography column with the mobile phase consisting of ethanol-ammonia-H_2O (8:1:1, v/v/v). The fractions were separately collected, concentrated and bio-assayed again. The active fractions were finally separated by a preparative HPLC column (LC-9101-JAIGEL-ODS) with the mobile phase of methanol-H_2O (7:3) at a rate of 3 ml/min, and monitored at 305 nm with a UV detector. Each single peak was collected separately, and the main active fraction picked out as the terminal sample and examined by analytical HPLC (LC-10AT) using various solvent systems to evaluate the purity.

The chemical structure of the terminal sample was determined by using spectroscopy methods. The ultraviolet spectrum within 190–400 nm was obtained with an UV-VIS 3010 spectrophotometer, and the infrared spectrum within 4000 nm^{-1}–400 nm^{-1} with a Tensor 27 Nicolet spectrometer. High resolution ESI plasma spectrometry was carried out using a

9.4T apex Q-FT-MS spectrometer under the following conditions: capillary 4000, dry gas 4.0 l/s, ion source temperature 180 °C, scan range 300～2000, syringe pump 1.5 ml/min. NMR analysis was done using a Bruker Avance Drx-500 Nmr spectrometer with N,N-Dimethylacetamide (DMAc) as solvent.

Results and discussion

The results of the bioassay showed that the active constituents were mainly collected from the mobile phase of 70 % ethanol in the case of the macroporous resin separation and the adjacent fractions of the eluates from the silica gel column. The active fractions resulted in 30 peaks on the first HPLC spectrum. The fraction corresponding to the peak appearing at the elution time of 57.87 min was identified as the active substance. A fraction of higher activity was obtained as the terminal constituent by two times continuous further HPLC separations, which corresponded to the peak at the elution time of 39.77 min on the last HPLC spectrum. Only one peak was detected for the terminal constituent by analytical HPLC, which revealed that the fraction contained a single compound. The relative area of the peak suggested a purity of the compound of up to 99.85 %, sufficiently meeting the requirement for structural analysis.

The UV spectrum showed that the active compound dissolved in methanol-water presented four typical absorbance peaks at wavelengths 281nm, 291nm, 305nm and 319nm, which is the typical characteristic of conjugated polyene chemicals. The IR ν_{max} 3593, 3493, 3280, 3017, 2978, 2940, 1716, 1634 and 1570 suggested the presence of following functional groups: N-H, -OH, $-CH_3$, $-CH_2$, C=C, C-H and C=O. High resolution FT-MS analysis confirmed that the molecular weight of the active compound was 665, and the molecular formula $C_{33}H_{47}NO_{13}$. In the ^{1}H-NMR spectrum, a group of peaks close to δ=6 displayed the hydrogen bound on the conjugated ester, and the peaks around δ=5 showed a –OH. The presence of –COOH and ester were shown by ^{13}C-NMR δ180 and δ165. The peaks near δ=130 revealed the polyene structure of the compound. By comparing with the data of UV, IR, MS and NMR reported by Kang *et al.*(2006), the active compound produced by *S. lydicus* A02 was identified as Natamycin.

Natamycin is a macrolide polyene fungicide widely used in pharmaceutical and food industry for the treatment of fungal infections and contaminations. As a natural biological preservative and anti-microbial additive for food, it has been used in more than 30 countries. The antibiotic was reported to be produced mostly by *S. chattanovgensis*, *S. natalensis*, *and S. gilvosporeus* (Wu *et al.*,2004). Our research revealed that Natamycin can be produced by *S. lydicus* and used for the control of fungal plant diseases. The result provided a new producing microorganism resource and an expanding application field for Natamycin, which is important for the future development and utilization of the fungicide.

References

Kang J; Wang M; Du L-X(2006). Extration and identification of natamycin from fermented broth. Pharmaceutical Biotechnology, **13**, 293-298

Pan Z-Y; Liu W-C; Qiu J-Y; Dong K-Y; Tian Z-F; Liu D-W; Liu X-M (2005). Control effect of the Actinomyces strains □-61 and A-21 to fusarium wilt and gray mold of vegetables. Acta Agriculturae Boreali-Sinica, **20**, 92-97

Wu J-G; Wang M; Yang D-J; Du L-X(2004). Screening of high natamycin-producing strain: *Streptomyces gilvosporeus*. Chinese Journal of Antibiotics, **29**, 332-334

Verticillium nigrescens; a non-aggressive wilt pathogen as a promising biocontrol agent for Verticillium wilt of eggplant and cotton

F T Gravanis, I K Vagelas, D G Natsiopoulos
Technological Education Institute of Larissa, Department of Plant Production, GR-41110 Larissa, Greece
Email: gravanis@teilar.gr

Introduction

Verticillium wilt caused by *Verticillium dalhiae* Kleb., is a well known serious disease of many economical crops and especially of cotton and eggplant. The fungus is widespread in most cotton cultivated areas as well as in horticultural areas in central Greece and is one of the greatest threats to cotton and greenhouse production. This severe pathogen was isolated many times from cotton plants showing symptoms of Verticillium wilt at several locations in central Greece. *Verticillium nigrescens* Pethybr., is a weak pathogen or a common soil inhabitant (Korolev & Katan, 1999). This pathogen was isolated from greenhouse tomato plants showing mild wilt symptoms. Cross protection of Verticillium wilt by *V. nigrescens* were reported for tomatoes by Gravanis & Xifilidou (2001). In the present study both organisms were used to determine cross protection effects on cotton (cv. 4S and Pandora) and eggplants (cv. Black Beauty) *in planta*.

Materials and methods

Both pathogens were grown on PDA plates which were incubated in darkness at 25°C for 20 days. The experiment was designed with seven treatments and 10 replications per treatment:

- Inoculation with *V. dahliae (Vd)* by dipping the roots of both plants in spore suspension (10^6 spores/ml) for 1 h.
- Inoculation with *V. nigrescsens (Vn)* by dipping the roots of both plants in spore suspension (10^6 spores/ml) for 1 h.
- Inoculation with *V. nigrescsens* and subsequently on the same day inoculation with *V. dahliae (Vn + Vd)* by adding spore suspension (10^6 spores/ml) in the pot (15 ml/pot).
- Inoculation with *V. nigrescsens* and after 4 days inoculation with *V. dahliae (Vn + 4Vd)* by adding spore suspension (10^6 spores/ml) in the pot (15 ml/pot).
- Inoculation with *V. dahliae* and subsequently on the same day inoculation with *V. nigrescsens (Vd + Vn)* by adding spore suspension in the pot, as above described.
- Inoculation with *V. dahliae* and after 4 days inoculation with *V. nigrescsens (Vd + 4Vn)* by adding spore suspension in the pot, as above described.
- Untreated plants (Control).

Wilt disease severity, stem height, diameter, fresh weight and dry weight of inoculated plants were assessed at the end of the experiment (after 47 days of incubation for cotton and 60 days of incubation for eggplant). All data were analysed by analysis of variance. A multivariate analysis was then performed to determine the cross protection effects of *V. dahliae* by *V. nigrescens* for both plants.

Results and discussion

The results of ANOVA and Multivariate Analysis showed that mild wilt symptoms occurred in both tested plants, when inoculation with *V. nigrescens* preceded *V. dalhiae* agreeing with Korolev & Katan (1999). Plants which were inoculated at the same day with

V. nigrescens before the inoculation with *V. dahliae* were not statistically different from the control plants, in disease severity, stem diameter, fresh and dry weight (Tables 1 & 2). Multivariate analysis also showed that when *V. nigrescens* was inoculated four days after *V. dalhiae,* significant wilt symptoms were observed and no cross protection occurred. This agrees with Melouk & Horner (1975), who observed cross protection in peppermint when inoculation with *V. nigrescens* preceded inoculation with *V. dahliae* by two days.

Table 1. Stem height and diameter by inoculation with *V. nigrescens* and *V.dahliae*

Tested plant	Cotton				Eggplant	
Tested cultivar	Pandora		4S		Black Beauty	
	Plants stem height and diameter (Diam.) mean values					
Treatment	Height (cm)	Diam (mm)	Height (cm)	Diam. (mm)	Height (cm)	Diam. (mm)
Control	45.1b	5b	49.0b	5.9b	23.6b	5b
Vd [1]	19.2a	3a	23.4a	3.3a	17.9a	4a
Vd+4*Vn*	27.6ab	3a	25.6a	3.6a	16.3a	4a
Vd+*Vn*	28.72b	3a	33.2ab	4.7ab	15.7a	4a
Vn [2]	42.9b	6b	43.2b	5.7b	22.1b	4a
Vn+4*Vd*	46.6b	6b	41.2b	5.2b	20.5b	5b
Vn+*Vd*	44.5b	6b	46.8b	6.2b	18.6ab	5b
P value $_{(0.05)}$	0.007	0.003	0.002	0.008	<0.001	0.011

Where: [1] *V. dahliae*, [2] *V. nigrescens*. Values in columns followed by the same letter show no statistical difference for p=0.05 according to Tukey multiple range test.

Table 2. Stem fresh and dry weights by inoculation with *V. nigrescens* and *V.dahliae*

Tested plant	Cotton				Eggplant	
Tested cultivar	Pandora		4S		Black Beauty	
	Plants stem fresh and dry mean values					
Treatment	Fresh (g)	Dry (g)	Fresh (g)	Dry (g)	Fresh (g)	Dry (g)
Control	28.6b	6.5b	25.8b	5.8b	10.1b	2.6b
Vd	7.5a	1.9a	4.1a	1.4a	8.1ab	1.7a
Vd+4*Vn*	9.4a	1.9a	6.4a	1.3a	6.2a	1.5a
Vd+*Vn*	11.4a	2.7a	13.4b	3.3b	6.1a	1.5a
Vn	25.3b	6.9b	19.7b	5.1b	9.8b	2.3b
Vn+4*Vd*	27.7b	7.8b	17.6b	4.9b	11.2b	2.5b
Vn+*Vd*	26.0b	6.3b	24.2b	6.1b	9.4b	2ab
P value $_{(0.05)}$	0.002	<0.001	0.003	0.008	0.001	0.001

References

Gravanis FT; Xifilidou S (2001). Pathogenicity of *Verticillium dahliae* and biological effect of *V. nigrescens* on Verticillium wilt of tomatoes. *Phytopathologia Mediterranea* **40**, 90-91.

Korolev N; Katan T (1999). Vegetative compatibility grouping in *Verticillium nigrescens* and *V. tricorpus*. *Mycological Research* **103**, 65-76.

Melouk HA; Horner CE (1975). Cross protection in mints by *Verticillium nigrescens* against *V. dahliae*. *Phytopathology* **65**, 767-769.

New *Pseudomonas* spp. strains with antimicrobial activities

C P Cornea, C Voaides, M Ciuca, I Grebenisan, G Popa, F Helepciuc
Faculty of Biotechnology, UASVM Bucharest, 59 Bd. Marasti, Bucharest, Romania
Email: pccornea@yahoo.com

Emergence of fungicide-resistant pathogens, health concerns for producers and consumers and phasing out of certain chemicals have accelerated studies for development of alternative practices to achieve more sustainable levels of agricultural production. Based on the large diversity of the microbial world, new strains of plant-associated rhizobacteria with antimicrobial activities could be isolated. In this respect, in a screening study of soil-originated bacteria performed in our laboratory, 68 bacterial strains, both Gram positive and Gram negative, were isolated. All these isolates were tested for antimicrobial activities against six fungal species: *Fusarium solani, F.oxysporum, Botrytis cinerea, Rhizoctonia* sp., *Alternaria spp., Aspergillus* spp., and against two *Erwinia amylovora* isolates.

The studies of interactions between *Pseudomonas* spp. strains and different pathogenic microorganisms were conducted on potato dextrose agar (PDA) and King's B media in Petri dishes. For characterization of the bacterial isolates, PCR based methods were applied using a programmable gradient thermal cycler (Eppendorf Mastercycler). A Promega kit was used to isolate bacterial genomic DNA. Different primers were used for characterization of the new bacterial strains.

Five individual 10-mer primers (Operon Technologies Inc.) were used, one at a time: OPA 9, OPP 07, OPR 17, OPA 3 and OPA 07. DNA was amplified in 40 cycles (94°C – 1 min., 36°C – 1 min., 72°C – 2 min.). The random primers pgs2 (GTTTCGCTCC) and pgs3 (GTAGACCCGT) were used for RAPD analysis (Kumar *et al.*, 2002). In order to identify the *P. putida* strains, a specific primer designated as REPc (5'GTA GGA GCG GGT TTA CCC G3') (Aranda-Olmedo *et al.*, 2002) was used. 16S-23S rDNA primers ITS1F (AAGTCGTAACAAGGTAG) and ITS2R (GACCATATAACCCCAAG) (Kumar *et al.*, 2002) were used to amplify a 560 bp segment specific to fluorescent *Pseudomonas* spp. The primers were synthesized and obtained from Dexter. PCR reactions were carried out in 20µl reaction containing 2 µl of 10x buffer (with 2.5 mM $MgCl_2$); 2 µl of 2mM dNTP mixture; 3 units of Taq DNA polymerase; 8 µl of H_2O, and 50ng of template DNA samples, using the following program: 92°C – 4 min.; 40 x (92°C – 1 min.; 48°C – 1 min.; 72°C – 2 min.); 72°C – 10 min. In order to distinguish between the strains that presented PCR products when ITS primers were used, restriction analysis was performed. The restriction analysis was conducted with Alu I and Taq I restriction enzymes in the following conditions: 5U enzyme, 1x buffer and BSA 1%, incubation at 37°C for 90 min. PCR products as well as restriction fragments were separated by gel electrophoresis using a 2% agarose gel in 0.5x Tris-borate-EDTA buffer at 5,0 V/cm and visualized in UV light.

Among the 68 bacterial isolates, 55 were Gram negative, and based on morphological cultural and biochemical aspects, 19 strains were found to belong to *Pseudomonas* genus. Inhibition of the fungal strains tested was observed with 22% of the pseudomonads, but only three of these inhibited a large range of fungi. These strains were designated as P10,P14 and P20 and they were used in various tests, both *in vitro* and *in vivo* (using green peppers as test plants), proving both antifungal and plant growth promoting properties.

The genomic DNA isolated was used for RAPD analysis with various 10-mer primers. The best results (polymorphism) were obtained with three primers belonging to OPA group: OPA 03, OPA 07 and OPA 09. The amplified banding profiles were clearly distinguishable; with sizes ranging from 2000 to 150 bp. Among the three primers studied, largest fragments were obtained with OPA 09 primer but the amplified banding pattern was complex for all the primers, which reflects a high degree of diversity among the *Pseudomonas* isolates. With OPP 07 and OPR 17 primers, no amplification or reduced number of PCR products were obtained. Based on the polymorphism obtained with OPA 09 primer, a dendrogram was constructed. The dendrogram that was calculated using Treecon program, based on Nei and Li formula, with unweighted pair group method with arithmetic mean (UPGMA) revealed the identity of two strains (P7 and P8) as well as a segregation of the 19 strains into different genotypic groups.

Because the classical microbiological tests allowed the identification of the genus of the new isolates, the identification to species level was performed by genetic techniques. In order to identify the *P. putida* strains, a specific primer designated as REPc (5'GTA GGA GCG GGT TTA CCC G3') (Aranda-Olmedo *et al.,* 2002) was used. Among the 19 isolates tested in our study, only two strains gave PCR products. Our results, compared with those reported in literature, suggest that the REPc primer allows the identification of *P.putida* strains through specific amplification products. No PCR products were obtained in the other strains.

When ITS type primers were used in experiments, the 16S-23S rDNA region from analysed *Pseudomonas* strains gave a single amplicon or multiple amplified products. For five strains, the size of the major amplicon is approximately 560 bp, which allowed the affirmation that all these strains belong to *Pseudomonas fluorescens* species. The other four strains also gave a single amplicon but its size was around 500 bp. To distinguish whether the strains presented a unique amplicon it was necessary to use restriction enzymes such as Alu I, MspI or Taq I and to examine the restriction pattern. The restriction profile, revealed after agarose gel electrophoresis, allowed the assumption that the strains designated as P1, P2, P10, P12 and P14 could belong to *Pseudomonas aeruginosa.* Other strains, P7, P8 and P9 share the same electrophoretic profile, similar to *P. fluorescens* ATCC. Similar results were obtained when primer pgs3 was used: a major band with size of 800 bp corresponding to a part of 23S rRNA gene was observed in almost all the strains tested.

Combining the results obtained by application of molecular techniques with those obtained by using the Biolog system of identification, we are able to say that the three strains selected in our experiments for their antimicrobial activities belong to the following species: *P.aeruginosa* (P10 and P14) and *P.fluorescens* (P20). But, even if their inhibitory properties are quite similar, intraspecific polymorphism was observed.

In summary, the selected *Pseudomonas* strains are very promising for use as biocontrol agents and for plant growth stimulation in tomato, pepper and cucumber.

Screening rhizobacteria for the biological control of *Fusarium oxysporum* and *Pythium ultimum* root rot of sorghum

A Idris, N Labuschagne, L Korsten
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
Email: ahmed.idris@fabi.up.ac.za

Introduction

Sorghum (*Sorghum bicolor*) is cultivated as the main staple crop in many parts of Africa. In Ethiopia, seedling death as a result of root rot caused by *Fusarium oxysporum* (Idris *et al.* 2007) and *Pythium ultimum* (unpublished) a few weeks after planting are commonly observed. Chemical control of these pathogens using fungicides is ineffective and unaffordable to many small scale farmers. There is a growing trend towards controlling such soilborne infections in a wide variety of crops using bacteria known as plant growth promoting rhizobacteria (PGPR) (Rangarajan *et al.*2003). The aim of the current study was to isolate bacteria from the rhizosphere of sorghum in Ethiopia and screen them for *in-vitro* and *in-vivo* antagonism against *F. oxysporum* and *Pythium ultimum*, identify the most effective isolates to species level and elucidate their modes of action.

Materials and methods

Soil sample collection and isolation of bacteria

Soil samples were collected from the rhizosphere of sorghum in Ethiopia and isolation of bacteria was conducted using the standard dilution plating technique on Nutrient agar (NA) and King's B (KB) medium. A total of 78 isolates were selected and maintained in nutrient broth and 15% glycerol at -70°C.

In-vitro antagonistic activity and root colonization

Bacterial inoculum for each isolate was adjusted to 10^8cfu/ml (OD_{550} = 0.5-1.0). *In-vitro* mycelial inhibition of *F. oxysporum* and *P. ultimum* was assessed by means of the dual culture assay on agar plates (Paulitz *et al.*, 1992).

Greenhouse evaluation of bacterial isolates

Bacterial isolates effective in the *in-vitro* tests were selected for the greenhouse evaluation. Eight sorghum seedlings each, pre-germinated in vermiculite, were transferred to 500 ml plastic pots containing steam pasteurised sandy loam soil. Each pot was drenched with suspensions (30ml of 10^8 cfu ml $^{-1}$) of the bacterial isolates twice a week apart. The treatments included: *Bacteria + pathogen*, *pathogen alone*, and *un-inoculated control* with three replications in a randomized block design. Root rot severity was recorded on a rating scale of 0-4, and the percentage disease suppression by the bacterial isolates was determined as follows (Villajuan Abgona *et al.*1996): % Disease Suppression = [(A-B) /A] x 100, where A = root rot severity due to pathogen alone, B= root rot severity after inoculation with the pathogen and bacterial isolates. Additional data recorded included root fresh and dry weight and necrosis of the leaves. Data were analyzed by analysis of variance (ANOVA) and means were compared by the least significance difference (LSD) test.

Identification of effective isolates and mode of action studies

Isolates effective in the *in-vitro* and greenhouse experiments were identified to the species level by means of the API system and 16S rDNA sequencing. During the mode of action

studies, production of antibiotic substances was detected from bacterial culture filtrates using the agar well diffusion assay. Siderophores were detected using the universal chemical assay on CAS agar plate; chitinolytic activity was evaluated by means of the chitin agar plate assay and induction of systemic resistance of sorghum against *F. oxysporum* by means of the split root technique.

Results

Application of isolates KBE5-7, KBE5-1, NAE5-5, all identified as *B. cereus* and KBE2-5, identified as *Bacillus* sp., resulted in 100% disease suppression of *Fusarium oxysporum* root rot in sorghum. A disease suppression ranging between 76 - 95% against *F. oxysporum* was rendered by seven other isolates (KBE9-1, KBE8-3, NAE6-2, KBE5-2, KBE5-4, KBE4-3 and NAE5-7), all of which were identified as members of the Genus *Bacillus*, and by one isolate of *Chromobacterium violaceum*.

Three isolates (NAE1-7, NAE7-1 & KBE6-1) identified as *B. subtilis* and *B. cereus*, respectively, resulted in disease suppression ≥ 75 %. Isolates which exhibited significant *in-vitro* and *in-vivo* suppression of *F.oxysporum* and *P. ultimum* showed a high level of root colonization ($\geq 10^5$ cfu g^{-1} of root) and modes of action including production of antibiotics and siderophores, chitinolytic activity and induction of systemic resistance.

Discussion

To our knowledge, this is the first report of effective biological control of soilborne diseases caused by *F. oxysporum* (Idris *et al.*2007) and *P. ultimum* in sorghum using rhizobacteria. The rhizobacterial isolates effective in this experiment were all isolated from the rhizosphere of the target crop i.e. sorghum. This approach is considered essential for the successful identification of biocontrol agents (Williams & Asher, 1996). Current trends in agriculture are focussed on the reduction of the use of fungicides and inorganic fertilizers. The use of PGPR inoculants as biocontrol agents is a promising alternative to chemicals for sustainable agriculture (Donate-Corea *et al.* 2004). The current study provides data on the occurrence of potential biocontrol bacteria in the rhizosphere of sorghum. However, to ensure the consistent performance of the bacterial isolates, this study warrants further field tests.

References

IdrisHA; Labuschagne N; Korsten L (2007). Screening of rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. Biological Control **40**, 97-106

Rangarajan S; Saleena LM; Vasudevan P; Nair S (2003). Biological suppression of rice disease by *Pseudomonas* sp. under saline soil condition. Plant and Soil **251**, 73-82

Villajuan-Abgona R; Kagayama K; Hyakumachi M (1996). Biocontrol of Rhizoctonia damping-off of cucumber by non pathogenic binucleate *Rhizoctonia*. European Journal of Plant Pathology **102**, 227-235

Williams GE; Asher MJC (1996). Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochliodes* on sugar beet seedlings. Crop Protection **15**, 479- 486

Donnate-Correa J; Leon-Barrios M; Perez-Galdona R (2004). Screening for plant growth promoting rhizobacteria in *Chamayecytisus proliferus* (tagasate), a forage tree shrub legume endemic to the Canary Islands. Plant and Soil **266**, 261-272.

Characterization of bacteriophages infecting *Xanthomonas oryzae* pv. *oryzae* to use as a biocontrol agent

Y H Lee, H Y Chang, D H Lee, S Heu, D-S Ra
Division of Plant Pathology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-707, Korea
Email:leeyh0@rda.go.kr

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae pv. oryzae*, is one of the most important bacterial diseases of rice worldwide. Although it can be controlled to a certain extent by agrochemicals, these chemicals have not always been effective, so alternative control measures are sought to improve control efficacy. Characteristics of *X. o.* pv. *oryzae*-infecting bacteriophages were studied in order to assess their potential as biocontrol agents.

Bacteriophages of *X. o.* pv. *oryzae* were isolated from the water of a paddy fields collected in Korea. Thirty-four phages were recovered from the collected samples and enriched, then stored in pettone sucrose media at 4°C until further use. To overcome any potential host-induced bias, the initial isolation and enrichment of the phages exploited a three-host system. The plaques of isolated phage were very clear and usually formed 6 hr after incubation with two types of plaque sizes.

To test for host range, 52 strains of bacteria were subjected to spot tests with the isolated phages. The strains to be tested were grown overnight in optimal PSA, LB, or KB. Three milliliters of the each molten soft agar was mixed with 100 µl of the each bacterial cells, which was then overlaid on the surface of the basal agar. Host range of the phage was determined by spotting a 3 µl of phage preparation on lawn cultures of the strains to be tested. The host range differed greatly according to the tested phages and *X. o.* pv. *oryzae* strains. There was no phage that could infect all of the tested 47 strains of *X. o.* pv. *oryzae* strains, and no *X. o.* pv. *oryzae* strains that is resistant to all of the tested 34 phages. The recently reported K3a race had a similar susceptibility to tested phages as that of K3 race. Interestingly, the phages which formed large plaques generally had a narrower host range than the other groups except for PX05-73L. Only the PX05-45M and PX05-60M had an extremely narrow host range.

Several bacteria which included *Burkholderia glumae, Pseudomonas syringae* pv. *tomato* DC3000, *Bacillus vallismortis* EXTN-1, *Burkholderia gladioli* K87, *P. fluorescence* were subjected to spot tests for evaluation of phages to form plaques on lawns composed of bacterial species other than *X. o.* pv. *oryzae*. None of these bacteria were susceptible to any isolated phages, indicating that these phages have a host specificity only to *X. o.* pv. *oryzae*.

High-titer phage liquid cultures were prepared and centrifuged at 10,000 g for 10 min to separate phage from the host cells. The supernatant containing the phages was centrifuged and the phage pellet was resuspended in sterile distilled water. One drop of the suspension was placed onto a nickel grid coated with formvar and negatively stained with 2% uranyl acetate or 3% sodium phosphotungstate. Specimens were viewed using a transmission electron microscope.

P6D-27

The *X. o.* pv. *oryzae* bacteriophages were placed into morphotype groups according to the method of Ackermann. Each phage investigated was one of the tailed phages between the A1 type of *Myoviridae* and B1 type of *Siphoviridae* families, which represent the largest part of all phage isolates. The head of *Myoviridae* had an elliptical, hexagonal outline approximately 56.7±2 nm in length and 60.8±2.8 nm in width. The width of *Siphoviridae* was 53.5±1.8 nm and 57.0±1.4 nm with variable lengths. The tail was attached to the head, which was approximately 84.6 nm and 145 nm in length in *Myoviridae* and *Siphoviridae,* respectively. The contracted stage was observed in *Myoviridae* family.

The effects of pH, heat and ultraviolet was on the stability of phages were investigated to check and increase the possibility of practical use in the field. Two ml of PS medium in tubes were adjusted to pH levels from 3 to 11 by the addition of 1N NaOH or 1N HCl, and enough phage was added to give a concentration of 1 X 10^3 pfu/ml. After 1 hour at 28°C, 0.1 ml of the sample was withdrawn and assayed for viable phage. Phages were rather stable between pH 5 and pH 10. The effectivity dropped sharply at pH below 4 and above 11. For the determination of the thermal inactivation, the phage suspensions were heated in a water bath at various temperatures for 1 hr. The plaque number sharply decreased when the phage suspension was incubated at 50°C and the phage was entirely inactive after incubation at 70°C. The stability of phages was checked at intensity of 5 $\mu W/cm^2$ of ultraviolet. More than 90% of the phages were inactivated after around five minutes of ultraviolet treatment, which will be the major obstacle for the practical use.

In the future the possibility of use as a biological agent will be tested with rice plants in greenhouse and field conditions.

Evaluation of non-chemical agents for control of black rot on grape vine (*Guignardia bidwellii*) in organic farming

E Koch
BBA, Institute for Biological Control, Heinrichstr. 243, 64287 Darmstadt, Germany
Email: e.koch@bba.de

D Molitor, B Berkelmann-Löhnertz
Geisenheim Research Center, Section Phytomedicine, Von-Lade-Str. 1, 65366 Geisenheim, Germany

B Loskill
BBA, Institute for Plant Protection in Viticulture, Brüningstr. 84, 54470 Bernkastel-Kues, Germany

Introduction

Originating from the Americas, black rot of grapes caused by the ascomycete *Guignardia bidwellii* (conidial state: *Phyllosticta ampelicida*) was introduced to Europe in 1885. Vineyards in Germany were seldom affected until 2002, when the disease was first discovered in the Mosel valley. In 2004 a severe epidemic occurred in parts of the Mosel wine growing area, and in recent years the disease has appeared regularly. The epidemiology of black rot is not fully understood, and the level of resistance in commercial varieties is not sufficiently known. While in conventional wine growing black rot is controlled by a number of chemical fungicides, organic farmers have little at hand to avoid or minimize the losses from this disease. Copper sprays at the low rates allowed in organic farming have proven ineffective. Therefore, agents for control of black rot specifically in organic farming are urgently needed. In 2006, a project integrating preventative measures, resistance management and disease control was initiated with the aim to improve the current situation of black rot control in organic farming. In the following, the results of greenhouse tests with alternative control agents, like commercially available bio-fertilisers, registered plant strengthening products and experimental agents of natural origin such as microorganisms and plant extracts are reported.

Materials and methods

The tested microorganisms were 34 bacteria and 18 filamentous fungi. The bacteria were cultured for 48 h in tryptic soy broth. The culture was centrifuged, and the supernatant added to molten potato dextrose agar (PDA) to produce amended PDA plates. Plates with PDA not amended served as controls. The plates were inoculated with *P. ampelicida* and after two weeks of incubation at room temperature mycelial diameters were measured and related to diameters on control plates. For efficacy testing on potted plants (see below) supernatants diluted with water (final concentration: 10%) were used. The biocontrol fungi were cultured in potato dextrose broth (PDB) for 12 days. The cultures were then centrifuged and the supernatants used for *in vitro* assays on agar plates as described for the bacteria. Furthermore, plant extracts prepared by Soxhlett extraction with ethanol, as well as a number of saponin-containing preparations (kindly supplied by Nor-Natur APS, Hvidovre, Denmark), the biofungicide Vegard 0.5% AS (kindly supplied by Kingbo Biotech, Beijing, China) and products of the official German list of commercial plant strengthening agents were included in this study. To evaluate their efficacy and that of selected microorganisms *ad planta*, a greenhouse assay on potted plants was performed.

The plant material used was two to three months old Riesling or Mueller-Thurgau plants raised from cuttings. All agents were applied 24 hours before inoculation. The five youngest unfolded leaves were inoculated with a suspension ($1x10^5$ per ml) of conidia of *P. ampelicida* obtained from sporulating cultures of the fungus on agar media. After incubation overnight in a humid chamber the plants were returned to the greenhouse. Disease was scored 14 - 21 days after inoculation based on % affected leaf area.

Results and discussion

On PDA amended with 10% culture supernatant, six of the 18 fungi and 14 of the 34 bacteria tested caused an inhibition of mycelial growth of *G. bidwelli* of >75%. At 1% culture supernatant, this level of inhibition was reached by only two of the bacteria and none of the fungi. In the plant tests performed so far, control of black rot by supernatants of all microorganisms tested was insufficient.

In the plant tests with fungicides approved for organic viticulture, sulphur showed a high efficacy. The efficacy of copper-containing products was generally lower. Products from the official German list of plant strengthening agents differed in performance. Satisfactory results were obtained with a rock meal product, two plant extracts and one product containing salts of phosphoric acid. All other tested substances had no or only minor efficacy against black rot.

Altogether, 27 ethanolic plant extracts were tested at the concentration of 5%. Most extracts provided at least some disease control. The most potent extracts were those from primrose roots (*Primula* sp.), ivy (*Hedera helix*), sage (*Salvia* sp.) and garlic (*Allium sativum*), all with efficacies of >90%. The extracts of some plants like lavender (*Lavendula* sp.), peppermint (*Mentha* sp.) and eucalyptus (*Eucalyptus* sp.) tended to promote the disease. In case of the extracts from primrose roots and ivy a similarly high efficacy was also recorded when these extracts were tested at the concentration of 0.5%.

Primrose and ivy both contain substantial amounts of saponins which are known to possess fungicidal properties and this may be related to the observed activity. We therefore included in the plant tests some saponin-containing preparations which in previous work have shown good activity against apple scab (personal communication by M Benker, KVL, Copenhagen, Denmark) and are currently being developed for commercial use. At concentrations of 5% and 0.5%, the preparations Quinoa Crude Liquid (extract of *Chenopodium quinoa)*, Nor-Spice Tea Liquid (*Camellia* spp.) and Norponin BS Liquid (*Yucca schidigera*) were similarly effective as the best plant extracts stated above. The same was true for the botanical fungicide Vegard 0.5% AS.

Following the screening on potted vines, selected agents were applied in an experimental vineyard in the Mosel wine growing area to evaluate their performance under conditions of natural infection. Based on the results, agents and strategies will be selected for practical use by organic vine growers.

Rice allelopathy and paddy weed management

C H Kong
Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China
Email: kongch@iae.ac.cn

Allelopathic rice (*Oryza sativa* L.) can release allelochemicals into the paddy which suppress the growth of neighboring weeds. Allelopathic rice seedlings appear to be able to detect the presence of *Echinochloa crus-galli* and respond by increasing certain allelochemicals. Allelopathic rice varieties, grown using integrated cultural management with a low dose herbicide, completely controlled the emergence and growth of most paddy weeds found and no grain yield reduction occurred. Therefore, this allelopathy-based technique for paddy weed management is worth exploring.

A few rice varieties or rice straw left in the fields after harvesting produce and release allelochemicals into the paddy in which suppress the growth of neighboring or successive plants. However, allelochemicals and mechanisms involved in rice allelopathy from living and dead plants are substantially different. Allelopathic rice PI312777 and Huagan-1 at their early growth stages released momilactone B, cyclohexene, and flavone into soil at phytotoxic levels through root systems (Kong *et al.*, 2004), but non-allelopathic rice Huajingxian and Lemont did not. Both allelopathic and non-allelopathic rice residues released similar concentrations and types of allelochemicals (momilactone B and lignin-related phenolic acids, *p*-hydroxybenzoic, *p*-coumaric, ferulic, syringic and vanillic acids) into the soil during residue decomposition to inhibit successive plants. In addition, living rice plants of allelopathic varieties appear to be able to detect the presence of inter-specific neighbours and respond by increasing certain allelochemicals (Kong *et al.*, 2006). The concentrations of the allelochemicals released from the allelopathic rice seedlings in soil increased dramatically when they were surrounded with *Echinochloa crus-galli*. The concentrations of the allelochemicals were over three-fold higher in the presence of *E. crus-galli* than in the absence of *E. crus-galli*. However, the same case did not occur in non-allelopathic seedlings surrounded with *E. crus-galli*. In addition to allelochemical exudation being promoted by the presence of *E. crus-galli*, allelopathic rice seedlings also increased allelochemical exudation in response to exudates of germinated *E. crus-galli* seeds or lepidimoide, an uronic acid derivative exuded from *E. crus-galli* seeds (Table 1).

Table 1 Chemical responses of rice seedlings to *E. crus-galli* associations

Treatment	Allelopathic variety		Non-allelopathic variety	
	PI312777	Huagan-1	Huajingxian	Lemont
A	23.4±5.2a	25.6±4.2a	4.3±0.6b	5.1±0.5b
B	71.6±6.7a	69.8±5.1a	5.3±0.7b	4.9±0.4b
C	64.6±5.9a	67.8±6.3a	4.5±0.5b	4.7±0.4b
D	45.3±4.7a	47.2±4.1a	5.0±0.9b	4.3±0.3b

Data are total amounts of allelochemicals (momilactone B, 3-isopropyl-5-acetoxycyclohene-2-one-1 and 5,7,4′-trihydroxy-3′,5′- dimethoxyflavone) in soil-grown rice seedlings at 3-leaf stage. Treatment, A, in the absence of *E. crus-galli*; B, in the presence of *E. crus-galli*; C, applied with the exudates of *E crus-galli* seeds; D, applied with lepidimoide (15 $\mu g\ g^{-1}$ soil).

Rice allelopathy can potentially be used to improve weed management in rice production. In the field, allelopathic rice PI312777 and Huagan-1 with integrated cultural management options including planting density, flooding depth and duration, and at a low dose herbicide (bensulfuron methyl, 25 a.i.g/ha, a third of the recommended dose) completely controlled the emergence and growth of most paddy weeds found. No grain yield reduction occurred, whereas, with non-allelopathic rice Lemont and Huajingxian, the grain yield reduction was 45-60% even with herbicide treatment (Table 2).

Table 2 Effect of rice varieties on paddy weeds under integrated managements in field

Treatment	Rice variety	Inhibition percentage of weeds		Rice yields (ton/ha)
		Grass	Broadleaf	
Integrated management without herbicide	PI312777	96.1±2.6a	61.0±8.3a	3.41±0.43a
	Lemont	48.7±6.7b	30.9±4.8b	2.17±0.09b
	Huagan-1	95.4±3.3a	64.0±5.5a	5.14±0.23c
	Huajingxian	42.4±2.8bc	38.0±3.3c	3.23±0.18d
Integrated management with herbicide *	PI312777	99.5±0.2a	87.4±5.2a	3.44±0.22a
	Lemont	58.7±2.9b	78.1±4.4b	2.73±0.11b
	Huagan-1	100±0.0a	90.3±3.8b	5.74±0.33c
	Huajingxian	55.4±3.1b	70.9±5.7c	4.05±0.15d

* Beron 100WP was applied at the rate 25 g/hm^2 at 6 days after transplanting.

Paddy weeds control using allelopathy could be incorporated to other allelopathic plants in field. However, weed-suppressive effects of these plant mulches are usually short-lived Weeds re-emerge and may cause considerable fieldwork. Whereas rice and weeds always simultaneously grow in paddies. Thus, the possibility of incorporating allelopathic traits into improved rice cultivars, which would show suppressive effects on the spontaneous growth of paddy weeds, would substantially reduce herbicide use. Although no commercial rice cultivars carrying allelopathic properties have been developed so far, it is now more widely accepted that cultivars with a built-in herbicidal system may be able to produce and release sufficient amounts of phytotoxins via root exudation to allow for substantial weed suppression, resulting in a reduction in the use of other weed management options (Belz, 2007).

This study shows that it is feasible to reduce herbicide dose in combination with allelopathic rice varieties under integrated cultural managements.

Acknowledgment
This work was financially supported by the National Natural Science Foundation of China (grant no.30430460).

References
Belz RG (2007) Allelopathy in crop/weed interactions–an update. *Pest Management Science* **63,** 308–326.

Kong CH; Laing WJ; Xu XH; Hu F; Wang P; Jiang Y (2004) Release and activity of allelochemicals from allelopathic rice seedlings. *Journal of Agricultural and Food Chemistry* **52,** 2861-2865.

Kong CH; Li HB; Hu F; Xu XH; Wang P (2006) Allelochemicals released by rice roots and residues in soil. *Plant and Soil* **288,** 46-57.

Herbicidal activity of 2′,3′-epoxyanisolactone and effect on metabolism of *Echinochloa crusgalli* L

S Q Wan, H B Lu
Key Lab of Pesticide and Chemical Biology, Ministry of Education, South China, Agricultural University, Guanghzhou 510642, China
Email: wanshuqing@scau.edu.cn

The herbicidal activity of extract from leaf of *Clausena lansium* with methanol and the active ingredients, 2′, 3′-epoxyanisolactone were assessed to *Echinochloa crusgalli* in laboratory. The active ingredient had been separated from the extract of C. *lansium* and the chemical structure was identified by means of ^{1}H-NMR and ^{13}C-NMR. The chemical name is 2′, 3′-epoxyanisolactone.

The result of toxicity assay indicated that the extract and 2′, 3′-epoxyanisolactone were very effective growth inhibitors against the weed *E. crusgalli*. The inhibiting effect to the root and the stem growth is as follows in table 1. The result of experiments also showed that the plant has the phenomenon of allelopathy to *E. crusgalli*. The allelochemicals, 2′, 3′-epoxyanisolactone synthesized by *C. lansium* can influence the growth and development of *E. crusgalli* and the mode of action to *E. crusgalli* may be reduced cell division and elongation.

Table 1: The toxicity of the extract and 2′,3′-epoxyanisolactone to *Echinochloa crusgalli*

Sample	Toxicity to root		Toxicity to stem	
	y=a+bx	IC_{50}(μg/mL)	y=a+bx	IC_{50}(μg/mL)
Extract	y=-1.7437+2.2144x	1110.10	y=0.2075+1.5225x	1415.30
Compound*	y=3.4033+1.7158x	8.52	y=3.1428+1.3969x	21.36

*:2′,3′-Epoxyanisolactone
Note: Data in a column with the same letter show no significant difference (*p=0.05*) (DMRT). The same for the following tables.

The result of physiological and biochemical assay showed that the compound could cause the weakness of vigor and functions of root systems to the weed. The compound decreased total amount of protein. Furthermore, treatment with a series of concentrations increased the quantity of amino acids in *E. crusgalli* (see Table 2).The results from the experiment indicated that the mechanism of inhibition to *E. crusgalli* may be the influence on the metabolism of protein, inhibiting the biosynthesis or inducing the decomposition of protein by the chemical and causing the accumulation of amino acids in tested plant.

Table 2: The effect of 2′,3′-epoxyanisolactone to *Echinochloa crusgalli* on metabolism of protein and amino acid

Concentration (mg/mL)	Protein Amount (mg/mL)	Reducing rate (%)	Amino acid	
			Amount of N(mg/mL)	Rate of N (%)
0.5	16.17±0.01e	75.91	1.3976±0.04a	1.6
0.25	22.53±0.02d	66.43	1.2972±0.34b	1.48
0.125	25.21±0.04c	62.43	1.2466±0.11b	1.43
0.0625	27.81±0.01b	58.57	1.1187±0.54c	1.28
0.03125	28.17±0.02b	58.03	0.9638±0.02d	1.1
CK	67.11±0.02a		0.8717±0.01e	1.0

The activity of peroxidase（POX）after treating the seedling with 2′,3′-epoxyanisolactone was increased and the activity of superoxide-dismutase (SOD) was also increased compared to the control, the quantity of malonaldehyde (MDA) increased compared to the control with a series of concentrations (see Table 3).

Table 3: The effect of 2′,3′-epoxyanisolactone to *E. crusgalli* on defensive enzymes and amount of MDA

Concentration（mg/mL)	POX		SOD		MDA	
	Enzyme activity		Enzyme activity		Amount	
	(FW) (U/g)	Rate of increase (%)	(FW) (U/g)	Rate of increase (%)	(μmol/g)	Rate of increase (%)
0.5	7.25±0.00a	140.98	1.39±0.06c	61.40	0.27±0.009a	246.40
0.25	7.17±0.01a	138.32	1.34±0.08c	55.12	0.18±0.0118b	132.81
0.125	6.64±0.01bc	120.71	1.19±0.07bc	38.05	0.13±0.0055c	66.57
0.0625	6.43±0.00ab	113.73	1.17±0.04bc	35.99	0.12±0.0058c	51.08
0.03125	5.15±0.07b	71.18	0.98±0.04ab	13.39	0.08±0.0044d	7.74
CK	3.01±0.10c		0.86±0.07a		0.08±0.0013d	

All the results of experiments mentioned above indicated that 2′,3′-epoxyanisolactone could act as a plant photosensitizer, since it actually contains 6 conjugated double bonds in its molecule. During excitation of 2′,3′-epoxyanisolactone by light, several activated states are attained. In the excited-state 2′,3′-epoxyanisolactone may interact with molecular oxygen transferring its energy to the oxygen. The resulting singlet oxygen can oxidize any molecule in its neighborhood and cause peroxidation of lipids to produce MDA as well as decomposition of protein. Defensive enzymes protect the cell from this destructive process. Under the condition of oxidization stress, defensive enzymes (POX & SOD) were activated

Further studies of 2′,3′-epoxyanisolactone will be on toxicity and mechanisms involved.

Phytotoxic effect of *Artemisia aucheri* on germination and growth of *Amarantus retroflexus*

H S Zadeh
Laboratory of Tropical and Subtropical and Ethnobotany, Coupure Links 653, B-9000 Gent, Belgium and Faculty of Natural Resources & Desert Studies, Yazd Universiy, Yazd, Iran
Email: hsodaee@yahoo.com

K Steppe
Department of Applied Plant Ecology, Faculty of Bioscience Engineering, Coupure Links 653, B-9000 Gent, Belgium

P Van Damme
Laboratory of Tropical and Subtropical and Ethnobotany, Coupure Links 653, B-9000 Gent, Belgium

Introduction

Weeds drastically reduce crop yields. Traditional methods for controlling weeds are time-consuming and labour intensive. Equally, imprudent use of chemical herbicides causes environmental problems. In sustainable agriculture, we need new strategies to improve weed and pathogen management. Allelopathy is a new method of weed control and could lead to reduced labour costs and increased efficiency, possibly without any adverse effects on the environment. Several *Artemisia* species have allelopathic potential (Escudero *et al.*, 2000, Modallal & Al-Charchafchi, 2006). One species, *A. aucheri*, is widely distributed in the desert area of Iran. Earlier field observations revealed that communities dominated by *A. aucheri* have a reduced density of other associated herbaceous species. Besides competing with other plants for nutrients, *A. aucheri* might be allelopathic and the presence of this plant may negatively influence growth of certain crops or weeds. Therefore, the present study was conducted to evaluate potential allelopathic effects of *A. aucheri* on the weed *Amarantus retroflexus*. The effect of aqueous extracts on germination and seedling development of *A. retroflexus* was evaluated. Further, *A. retroflexus* was grown in soil amended with plant material of *A. aucheri*, and the effect on plant growth was analysed.

Materials and methods

16 g fresh above-ground material of *A. aucheri* was cut into 1 to 2 cm parts. The plant material was put in a flask containing 100 ml distilled water, and the flask was placed for 24 h on an orbital shaker (Edmand Bahler, VKS-75). The extract was filtered through two layers of cheese cloth and centrifuged at 5000 rpm for 20 min. The supernatant was filtered again through two layers of Whatman No.1 filter paper. The extract was diluted with sterile distilled water to give final concentrations of 4, 8, 12 and 16 % (w/v). Seeds of *A. retroflexus* were surface-sterilized with 5 % sodium hypochlorite for two minutes, rinsed five times with distilled water and dried between two paper towels. To determine the effect of the *A. aucheri* extract on seed germination, 25 seeds of *A. retroflexus* were placed in Petri dishes with 9-cm Whatman no.1 filter paper, containing 5 ml of each *A. aucheri* aqueous extract or distilled water (control). The Petri dishes were placed in an incubator at 25 ± 1°C. The number of germinated seeds was recorded daily. After eight days the final germination percentage, mean percentage and mean period of final germination (MPFG) were calculated. The same procedures were also followed with 25 germinated seeds to

evaluate the effect of the aqueous extract of *A. aucheri* on seedling growth of *A. retroflexus*. The treated seedlings were placed in an air conditioned room at 25 ± 1°C with a 16/8 h (light/dark) photoperiod. After eight days root and shoot (coleoptile) length and seedling dry weight were measured.

In another set of experiments, shade-dried powder of above-ground material of *A. aucheri* was mixed with sandy loam soil (3, 6 or 9 g dry weight per 1000 g soil). The mixture was filled in plastic pots, which were placed in the greenhouse. Controls were pots filled with soil without amendment. Fifty ml tap water were added to each pot, and after one day, 15 seeds of *A. retroflexus* were sown in each pot. The plants were harvested 30 days after planting and leaf area, root length, plant height and dry weight were measured. Treatments were arranged in a randomised complete block design with three replicates. Data were subjected to an analysis of variance and the means were compared using least significant difference (LSD) at the 5% probability level.

Results and discussion

Aqueous extracts from fresh biomass of *A. aucheri* had a strong inhibitory effect on seed germination of *A. retroflexus* and significantly increased MPFG. At the highest concentration tested (16%), a reduction of germination by 98 % was observed when compared to the control. The MPFG increased progressively with increasing extract concentrations. At 4, 8, 12 and 16 %, the MPFG increased by 25, 87, 109 and 182 %, respectively compared to the control. Both root and shoot lengths were severely affected and the effect was more drastic on root length than on shoot length. At the concentration of 16 %, root and shoot length decreased by 73 and 43 %, respectively. The dry weight of seedlings was always significantly reduced, except at the lowest extract concentration (4%). At the highest concentration, the reduction in seedling dry weight was approximately 51 %. Incorporation of powdered above-ground plant material of *A. aucheri* affected the growth of *A. retroflexus* at all concentrations. Leaf area, root length, plant height and biomass were lower in soils with amendments compared to amendment-free soils. The amount of amendment added and the resulting inhibitory effect on plant growth were positively correlated. For example, the leaf area and root length declined progressively with increasing concentrations of soil amendment. Addition of 9 g powdered plant material from *A. aucheri* added to 1000 g soil reduced the leaf area and root length of *A. retroflexus* by 73% and 47%, respectively, compared to non-amended soil. Amendments of 3, 6 and 9 g per kg soil reduced dry the weight of *A. retroflexus* by 58, 73 and 81%, respectively. In conclusion, a bioassay with aqueous extracts or plant residues of *A. aucheri* demonstrated an allelopathic effect towards *A. retroflexus*. Germination and growth parameters of *A. retroflexus* were decreased, and the effect was concentration-dependent. The observed effects may have potential for practical use, providing an example for biological weed control based on natural plant compounds.

References

Escudero A; Albert M; Pita J; Garcia F (2000). Inhibitory effects of *Artemisia herba-alba* on the germination of the gypsophyte *Helianthemum aquamatum*. Plant Ecology **148,** 71-80.

Modalla NB; Al-Charchafchi FMR (2006). Allelopathic effect of *Artemisia herba-alba* on germination and seedling growth of *Anabasis setifera*. Pakistan Journal of Biological Science **9,** 1795-1798.

Use of fungal pathogens of chromolaena (*Chromolaena odorata* (L) K&R) for its biological control

A Naseema, N S Saritha, A Safeena, K K Sulochana
Department of Plant Pathology, College of Agriculture, Vellayani, 695522, Kerala, India
Email: naseemadr@yahoo.com

Chromolaena (*Chromolaena odorata* (L) K&R; Asteraceae), popularly known as Eupatorium weed / Siam weed, native of South and central America, is an invasive obnoxious weed. It is known to suppress growth of plantation species *viz.*, teak, dalbergia, eucalyptus and crops such as coconut and arecanut. The planned introduction of biocontrol agents would offer a most economic and environmentally compatible and sustainable method for long term control of this exotic weed. The use of microorganisms in the biological control of weeds is gaining importance now. Chromolaena is known to be infected by several plant pathogens: *Cionothrix praelonga* and *Cercospora eupatorii* (Barreto & Evans, 1994), *Septoria ekmaniana* and *Mycovellosiella perfoliata* (Elango *et. al.*,1993), *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Aureobasidium pullulans* (Prashanthi & Kulkarni, 2005). The present investigation is aimed at screening fungal pathogens of chromolaena for its biological control.

A survey was conducted in the Western Ghat area of Kerala state, India, to identify various fungal pathogens of chromolaena. The fungi isolated were maintained in PDA slants and the pathogenicity was proved by inoculating on healthy chromolaena plants. The extent of damage caused by the pathogenic fungi was calculated using a score chart. Host range studies with 57 plants including both common weeds and cultivated plants belonging to 37 families were carried out. The dosage of inoculum of the pathogens required for effective eradication of the weed was also determined. The spore concentrations were fixed at three levels. Based on the intensity of infection a disease index was calculated.

During the survey 19 fungi were isolated of which 11 were pathogenic, v*iz.*, one isolate each of *Alternaria alternata*, *Corynespora cassiicola*, *Curvularia prasadii*, *Drechslera* sp., *Fusarium moniliforme* var. *anthophilum*, *Lasiodiplodia theobromae*, three isolates of *Curvularia lunata* and two non-sporulating fungi. These fungi produced varying types of symptoms on the weed. *A. alternata* produced minute reddish brown spots with yellow halos on the leaves that coalesced to form large brown spots with faint yellow halos and later enlarged to form irregular lesions covering the major area of the leaves within four to five days. *C. prasadii* produced large water soaked spots that later changed to black lesions within five to six days of inoculation. The intensity of infection produced by the pathogens of chromolaena was recorded (Table1). *A. alternata* showed the maximum intensity of infection (86%), followed by C. *prasadii* (85%) under laboratory conditions. The least disease (36% infection) was recorded for C. *cassiicola*.

In order to find out the safety of the efficient pathogens on non-target plants, detailed host range studies of the efficient pathogens were performed. *A. alternata* was pathogenic to none of the cultivated plants, but to seven weed plants *viz.*, *Tridax procumbens*, *Vernonia cinaria*, *Synedrella nodiflora*, *Emilia sonchifolia* and *L. camara*. Similarly, *C. prasadii* could not produce symptoms on any cultivated plant. However, it was pathogenic to five weed plants viz., *Vernonia cinaria*, *Emilia sonchifolia*, *Eclipta alba*, *Commelina bengalensis* and *Lantana camara*. An inoculum concentration of 4.5 x 10^{11} spores ml^{-1} of

A. alternata resulted in maximum disease index of 88.4 %. Similarly, *C. prasadii* at 4.5 x 10^{11} caused 83.0 % intensity of infection.

Table1. Extent of damage produced by pathogens of chromolaena

Pathogen	Infection (%)
Alternaria alternata (Fr.) Keissler	86
Corynespora cassicola (Berk. and Curt.) Wei.	36
Curvularia clavata Jain (isolate 1)	82
C. clavata Jain (isolate 2)	77
C. clavata Jain (isolate 3)	79
C. prasadii R.L & B.L. Mathur	85
Drechslera sp.	60
Fusarium moniliforme var. *anthophilum* (A.Braun) Wollenw and Reink)	75
Lasiodiplodia theobromae (Pat.) Griffon and Maubl	43
Non sporulating fungus (isolate 1)	76
Non sporulating fungus (isolate 2)	66

The results of the study indicate that A. *alternata* and C. *prasadii* are candidates for biocontrol agents of chromolaena, since they caused a high intensity of infection and had a narrower host range among the cultivated plants than the other pathogens studied.

References

Barrreto R W; Evans H C (1994). The mycobiota of the weed *Chromolaena odorata* in Southern Brazil with particular reference to fungal pathogens for biological control. Mycological Research **98**, 1107-1116

Elango D E; Holden A N G; Prior C (1993). The potential of plant pathogens collected in Trinidad for biological control of *Chromolaena odorata* (L.) King & Robinson. International Journal of Pest Management **39,** 393-396

Prashanthi S K; Kulkarni S (2005). *Aureobasidium pullulans,* a potent mycoherbicide for biocontrol of eupatorium [*Chromolaena odorata* (King & Robinson)] weed. Current Science **88**, 18-21

Biological control of water hyacinth with a mycoherbicide

A Naseema, M S Ancy, R Praveena
Department of Plant Pathology, College of Agriculture, Vellayani, 695522, Kerala, India
Email: naseemadr@yahoo.com

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] is a pernicious aquatic weed infesting more than 200,000 ha of water surface, causing concern in 98 out of the 246 districts in India. Inundative and classical control approaches using plant pathogens have been proposed to mitigate the impact of this weed. Water hyacinth is known to be infected by the pathogens *Alternaria eichhorniae* (Nagraj & Ponnappa, 1970) and *Fusarium pallidoroseum* (Santhi & Naseema, 1995). The objective of the present study was to develop a mycoherbicide using the pathogens of water hyacinth for its biological control.

A survey was conducted to document the pathogens of water hyacinth in the water ways of the Kerala State, India. The observations on the nature of symptoms and time taken for their development were recorded. An experiment was conducted to select the most promising pathogens by analyzing the intensity of infection produced by them and their metabolites. The cell free metabolites were prepared by collecting the filtrate of the fungi grown in Czapek Dox broth after passing through a 0.4 μm Sartorius membrane filter. The extent of damage was calculated using a 0-6 score chart. A detailed host range study of the efficient fungi and their metabolites was carried out with 53 species of cultivated plants and 54 species of weed plants belonging to 47 families. An experiment was conducted to standardize the dosage of inoculum of the promising pathogens *viz*., *A. eichhorniae* and *F. pallidoroseum* individually and in combination for effective destruction of the weed. A talc based combination product of *A. eichhorniae* and *F. pallidoroseum* was prepared by mixing mycelial mats from both fungi with 10% talc and 1% carboxy-methyl-cellulose. The efficacy of this preparation was tested on water hyacinth in the glass house and in cement troughs ($1 \times 1 \times 0.4$m). The shelf life of the formulation was tested by storage under room conditions (28 ± 2°C).

The survey to identify the pathogens of water hyacinth yielded 14 pathogenic fungi. Of these, *Curvularia lunata, Colletotrichum gloeosporioides, Fusarium pallidoroseum, F. moniliforme, F. oxysporum* and *Myrothecium advena* were observed consistently in all the areas surveyed. Based on the time taken for development of symptoms by the pathogens and their metabolites, the pathogens were grouped into three categories: I- highly virulent, II-moderately virulent and III-avirulent. *M. advenae* and *F. pallidoroseum* (isolate 3) were categorised under group I as they developed symptoms within five days in the form of large lesions leading to complete drying up of the plant. Group II included *A. eicchorniae, F. moniliforme, F. oxysporum* (isolate 1 and 2) and *F. pallidoroseum* (isolate 1 and 2), as they developed symptoms within seven days. Water hyacinth multiplies at a very fast rate and can double its biomass in 10 days. Therefore, a fungus which is effective in killing the weed in less than 10 days is potentially suited as an effective biocontrol agent.

The pathogenic fungi varied in their extent of infection caused to water hyacinth (9.9 to 58.8%). The maximum damage was recorded for *M. advena*, followed by *F. pallidoroseum* (53.3%). The extent of damage caused by *A. eichhorniae* (48.9%) was comparable to that of *F. moniliforme* (50.1%). The cell free metabolites of these fungi showed significant differences in the intensity of damage they caused on water hyacinth. Maximum intensity

(89.0 %) was produced by *M. advena,* followed by *F. pallidoroseum* (isolate 3) (49.8 %). Based on the extent of damage caused by cell free metabolites, *M. advena* and *F. pallidoroseum* (isolate 3) were assigned to group I, which took less than five days to develop symptoms.

Safety to non target plants is important for the use of a plant pathogen as mycoherbicide. Host range studies revealed that *M. advenae* was pathogenic to 27 cultivated and 45 weed plants. This broad host range limits its usefulness as a potential biocontrol agent of water hyacinth. *F. pallidoroseum* produced symptoms on four cultivated and 20 weed plants. Its host range was narrower compared to *M. advena* and even on susceptible plants it could initiate symptoms only when the host surface was injured. *A. eichhorniae* did not infect any of the cultivated plants, however it was pathogenic to three weed plants tested. The narrow host range of *A. eichhorniae* was reported by Nagraj& Ponnappa (1970) and Shabana *et. al.* (1995).

The combined application of *A. eichhorniae* and *F. pallidoroseum* resulted in a higher intensity of infection than application of either pathogen alone. Also, the time required for symptom development was only five to six days, less compared to the application of the single pathogens. The toxin produced by *F. pallidoroseum* might have weakened the plant and made the host more vulnerable to the infection by *A. eichhorniae*. There are reports on the use of more than one pathogen for effective weed control (Charudattan, 1986).

The talc based formulated product at 10 and 5% caused an intensity of infection of 93.2 and 81.2%, respectively, 10 days after spraying. This product retained the viability of spores up to two weeks under room temperature (28 ± 2°C). After this period a sudden decline in spore viability was observed.

In conclusion, *A. eichhorniae* and *F. pallidoroseum* satisfy many of the requirements of an organism for weed control, such as narrow host range, reasonably high infectivity, culturability and safety for the user and environment. Our study indicates that these pathogens have potential for large field application, both individually and in combination.

References

Charudattan R (1986). Biological control of showy crotalaria with two fungal pathogens. Weed Science Society of America Abstract **26**, 51

Nagraj T R; Ponnappa K M (1970). Blight of water hyacinth caused by *Alternaria eichhorniae* sp. nov. Transactions of the British Mycological Society **55**, 123-130

Santhi K S; Naseema A (1995). Seasonal occurrence of fungi on water hyacinth [*Eichhornia crassipes* (Mart.) Solms] in Kerala. Journal of Tropical Agriculture **33**, 102-104

Shabana Y M; Charudattan R; Elwakil M A (1995). Identification, pathogenicity and safety of *Alternaria eichhorniae* from Egypt as a bioherbicide agent for water hyacinth. Biological Control **5**,123-125

Allelochemicals derived from tropical plants and syntheses of their derivatives for plant protection

T D Xuan, S Tawata
Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Okinawa, 903-0213, Japan
Email:g056003@agr.u-ryukyu.ac.jp

Objectives

Bioactive compounds from plants have been a promising source for weed and pest management. They can replace unsafe agricultural products, help to ease environmental pollution and human health problems attributed to the overuse of synthetic agrochemicals. Okinawa in the south of Japan, has a rich plant ecosystem, of which many tropical species are known to possess pharmacy and pest suppression properties: Alpinia (*Alpinia zerumbet*) and Leucaena (*Leucaena leucocephala*) are among the most well-known and widely distributed. Leucaena has great potential as animal feed for high protein content, but its use is limited because of the non-protein amino acid mimosine (β-[N-(3-hydroxy-4-pyridone)]-α-aminopropionic acid) which causes diseases in animals when ingested. Alpinia has fast growth and strong resistance to pests and diseases and has been a traditionally important and economic plant in Okinawa. Bioactive substances including essential oils (majority of which are terpenes), polyphenols, phenols, lactones, and amino acids. Essential oils, dihydro-5,6-dehydrokavain (DDK), and mimosine suppressed weeds and fungi. However, the direct use of these natural products in fields is difficult because of instability and cost. Over a period of 20 years, we have developed different techniques to isolate large amounts of secondary metabolites from tropical plants in Okinawa, which are simple and economically convenient. In addition, many derivatives of these compounds have been synthesized in our laboratory and examined for their biological activities.

Materials and methods

Essentials oils and DDK were purified from Alpinia. Mimosine was isolated from Leucaena. Simple purification methods developed in our laboratory allowed a great amount of these substances to be obtained from Alpinia and Leucaena. The oils were extracted through a distillation process and then were extracted with diethyl ether. The composition and quantity of the essential oils were determined by GC-MS. The plant material (mainly leaves) of Alpinia were boiled for 1 h and extracted with hexane to give a mixture of DDK and 5,6-dehydrokavain (DK). Pure DDK was then obtained by thin layer chromatography (TLC). A complex of ion-exchanged resin was applied to purify mimosine from Leucaena.

A number of compounds derived from DDK, mimosine, and major essential oils in Alpinia were synthesized in our laboratory. For mimosine derivatives, major synthesized compounds were belonging to propionates and 2-aminoalcohols. For DDK, the methoxy group in the lactone ring was replaced by hydroxyl and other different functional groups. Mimosine derivatives were synthesized from 2-hydroxypyridine (material A) and 4-hydroxypyrine (material B), whereas material C acrylates. Derivatives of DDK was synthesized from HMP (5-hydroxy-6-methyl-2H-pyran-2-one) and derivatives of benzoic acid. Isothymol, thymol, and eugenol in the essential oils of Alpinia possess strong antifungal activity against plant pathogenic fungi. Although these compound are volatile in the nature, they were converted into nonvolatile phosphorothionates by reaction with thiophosporic agents.

Chemical structures of the natural products and their derivative compounds were confirmed by analytical instruments including mass spectra, IR, ^{1}H-NMR, and ^{13}C-NMR. Their antifungal, herbicidal, and insecticidal activities were examined and compared with those of the oils, DDK, and mimosine.

Results

Among synthesized compounds, chrolo-3-(2-oxohydoropyridyl) and chrolo-3-(4-oxohydoropyridyl) propionates exhibited maximum herbicidal activity against growth of *Brassica rapa* (50-70% of inhibition). On the other hand, lengths of radicle and hypocotyls were either promoted or inhibited by the propionates A3, A4, A11, B5, B6, B11, and B12. The other propionates reduced growth of *B. rapa* by lower magnitude (20-40%). The chloric group in the two propionates A2 and B2 may be responsible for the greater herbicidal activity than other compounds. However, none of these mimosine derivatives could exert stronger herbicidal activity than mimosine, which showed 80-90% of inhibition. For antifungal activity, the compounds A1, A2, A11, B6, and B8 were the most inhibitive against both *Rhizoctonia solani* and *Schlerotium dellfinii* (50-70% of inhibition), whereas there were 5 propionates B3-B5, B11, B12 that stimulated growth of the two fungi up to 20%. Growth of *R. solani* and *S. dellfinii* were either stimulated or suppressed by compounds A3 and A4. The other propionates exerted fungal activity by 10-40%. The compounds chloro-3-(2-oxohydoropyridyl) and chloro-3(4-oxohydoropyridyl) propionates (A2 and B2) showed good antifungal activity, whereas the chloro-3(4-oxohydoropyridyl) propionate exhibited weak suppression against growth of *R. solani*. The 2 compounds A2 and B2 showed the most potential among the synthesized propionates of herbicidal and antifungal activities. Mimosine did not show any effect against *S. dellfinii*, but inhibited growth of *R. solani* by 30%. The antifungal strength of these synthesized propionates was greater than that of mimosine, with the exception of A4, A8, B4-B6, B11, and B12 propionates.

The insecticidal activity of DDK and its DDK-OH showed poor insecticidal activity when tested at concentrations of 1.0 and 0.1 mg on filter paper, whereas compound demethyl derivative showed the strongest activity of 93%, followed by the synthesized diethyl by 50% and diphenyl derivative was the least activity. For antifungal activity, DDK showed greater strength than the dimethyl derivative against *Pythium* sp. However, the dimethyl compound exhibited the strongest activity (91.0%) against *C. rolfsii*, followed by DDK. The diphenyl derivative was the lowest of fungal suppression. The activity of these DDK derivatives could be ranked by this order: dimethyl>diethyl>diphenyl. The biological activities varied among derivatives of essential oils, DDK, and mimosine. Some compounds showed herbicidal and antifungal strength greater than their parental substances. These derivatives may be exploited for development of bioactive pesticides. Further derivatives from essential oils, DDK, and mimosine with different functional groups are being synthesized in our laboratory.

Acknowledgement

The authors express sincere thanks to the Grand-in-Aid for Scientific Research (No. 18880020) by Japanese Society for the Promotion of Science (JSPS), as well as the support from the Weed Society of Japan to Dr Tran Dang Xuan.

Comparison of different drying techniques for potential biocontrol agents

I L Bisutti, D Stephan
Centre for Research and Development of Crop Protection with Low Environment and Consumer Health Impact (SAFECROP), % Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Heinrichstr. 243, D-64287 Darmstadt, Germany
Email: Isabella.Bisutti@iasma.it, d.stephan@bba.de

A P Matos da Silva, R Nawrotzki
Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Heinrichstr. 243, D-64287 Darmstadt, Germany

Introduction

Formulation is an important step in the development of microbial biocontrol agents. A key problem is the development of stable formulations. Stability can be achieved by drying the micro-organisms. For commercial production, freeze-drying, spray-drying and fluid bed-drying are the most common drying techniques. Depending on the particular micro-organism and the desired formulation, appropriate processes have to be developed. Here we report results on optimization of three different drying techniques for three different micro-organisms. For the drying experiments, a laboratory spray-dryer (Mini Spray Dryer, Büchi, Switzerland), a laboratory freeze-dryer (Advantage EL, VirTis, USA) and a laboratory fluid bed-dryer (STREA-1, Aeromatic-Fielder, Switzerland) were used. A spray-drying procedure was developed for submerged spores of the entomopathogenic fungus *Metarhizium anisopliae* isolate Ma 97. A freeze-drying procedure was developed for the antagonistic bacterium *Pseudomonas flourescens* strain Pf153 (produced in liquid culture) and a procedure for fluid bed-drying was developed for conidia of the antagonistic fungus *Trichoderma harzianum* strain T39 produced by solid state fermentation.

Lyophilization

Freeze-drying or lyophilization is a drying technique in which the suspension medium is crystallized at low temperatures and thereafter sublimed from the solid state directly into the vapour phase. The goal of freeze-drying is to create a product with good shelf-stability and which is unchanged after its reconstitution with water. Although freeze-drying is expensive, it is one of the most conservative industrial drying techniques. It is suitable especially for fragile vegetative cells, like those of *P. fluorescens*. Therefore, research on the development of freeze-drying protocols was carried out using *P. fluorescens* strain Pf153. To optimize the freeze-drying process, different freezing and drying temperatures and protectants were compared. Viability, storability and biocontrol efficacy of freshly produced and dried cells were evaluated. By optimization of the freeze-drying process the viability (number of living cells) was raised from 10% to nearly 100%. Storability tests were carried out with the selected protectants (skimmed milk, sucrose, glucose, lactose and acid lignin). The highest viability after storage was obtained by formulating Pf153 in lactose. When the biocontrol efficacy of freshly produced and freeze-dried Pf153 was compared (test with *Botrytis cinerea* on detached leaves of *Vicia faba*), no significant differences were obtained. The dried material can easily be milled and resuspended in water. However, as a result the product becomes dusty, so that further formulation steps (e.g. granulation) have to follow.

Spray-drying

Spray-drying is a comparatively cheap industrial drying technology: large quantities of material can be quickly dried by this technique. The disadvantage is that generally high outlet temperatures are required. This technique is therefore suitable particularly for thermo-tolerant micro-organisms *e.g.* spore forming bacteria like *Bacillus thuringiensis*. Depending on the nozzle adjustments, the dried product is a very fine powder, and further formulation steps are necessary. Stephan & Zimmermann (1998, 2001) showed that after optimisation of the protectants and temperature adjustments the desiccation sensitive submerged spores of the entomopathogenic fungus *M. anisopliae* can be spray-dried without loss of viability and efficacy. Skimmed milk powder in combination with sugars resulted in a high viability with outlet temperatures of up to 50°C. Inlet temperatures of up to 135°C did not negatively influence the viability. After spray-drying the resulting fine powder could be suspended in oil or water. When suspended with protectants in oil, the spores became microencapsulated by the former. The mean diameter of the particles was 14.2µm. Therefore, spray-drying can also be a suitable technology for the development of oil-based Ultra Low Volume formulations. In water the protectants dissolved, and the micro-organism became visible.

Fluid bed-drying

In fluidised beds a product can be dried, granulated and coated. A great variety of formulations can be developed by fluid bed-drying. Extruder granulates, granules, coated seeds, pellets or tablets are some examples. The ready product particles are large in comparison to spray-dried material. In general the product is dust-free, which is an important advantage for the protection of the user. In comparison to spray-drying the drying temperature is lower in fluid bed-drying. Therefore, this method should be more suitable for thermo-sensitive micro-organisms. When conidia of *Trichoderma harzianum* produced by solid-state fermentation were fluid bed-dried, the viability after drying was mainly influenced by the selected protectants and the outlet temperature of the heated air. After optimizing these parameters, a viability of approximately 80% was achieved. In bioassays on detached potato leaves there was no significant difference in efficacy between dried or undried conidia of *T. harzianum*. The material used as carrier (e.g. sugars or millet seeds) did not significantly influence the viability of the product.

Conclusions

The results demonstrate that all of the drying techniques have their specific limitations. Therefore, depending on the micro-organism, an appropriate drying technique has to be selected and optimized to get a stable formulation to fulfil the quality criteria of biocontrol agents.

Acknowledgement

This research was partly supported by SafeCrop Centre, funded by Fondo per la Ricerca, Autonomous Province of Trento.

References

Stephan D; Zimmermann G (2001). Locust control with *Metarhizium flavoviride*: Drying and formulation of submerged spores. In: Koch E; Leinonen P (eds.), *Cost Action 830 - Microbial Inoculants for Agriculture and Environment - Formulation of Microbial Inoculants*, European Commission, EUR 19692, p. 27-34

Stephan D; Zimmermann G (1998). Development of a spray-drying technique for submerged spores of entomopathogenic fungi. Biocontrol Science and Technology **8**, 3-11

Micro-organisms to protect our crops and soils: a proposal to evaluate their environmental safety

B J W G Mensink, J W A Scheepmaker
RIVM (Rijksinstituut voor Volksgezondheid en Milieu-Stoffen Expertise Centrum; National Institute for Public Health and the Environment-Expertise Centre for Substances)
PO Box 1, 3720 BA Bilthoven, The Netherlands
Email: hans.mensink@rivm.nl

Introduction

Micro-organisms used for crop protection are allegedly safe when applied as prescribed. However, there is consensus that crop protection products with fungi and bacteria always require a proper pre-market safety evaluation. This is not only in view of *potential* toxicity, infectivity and pathogenicity of the micro-organism to other organisms, but also because of the possible effects of microbial contaminants and co-formulantia (spreader, sticker).

A proper safety evaluation is of particular importance as microbial crop protection products (MCPP) will be increasingly used for sustainable crop protection worldwide. Also, as the efficacy of the current MCPPs is not always satisfactorily, there is a trend to improve these products. An improved efficacy may enlarge the potential environmental impact, particularly in case of a broad range of target insects.

How should regulatory managers from industries, regulators from governments, or environmental scientists deal with such risks? Scientific and technical guidance on the safety evaluation for regulatory reasons is scarce and therefore we have developed an environmental risk decision tree in cooperation with the Dutch Ministry of Environment and the National Board for the Authorisation of Pesticides. This decision tree enables stakeholders to assess whether the environmental risks are acceptable, taking into account the efficacy, characterisation, identification, use pattern, emission, exposure and environmental effects of MCPPs.

Methods

A decision tree has been used as the format for regulatory guidance. Risk criteria and descriptions as embedded by the EU were the starting point for this tree. However, in view of harmonisation, descriptions and procedures of the North American Free Trade Association and the Biopesticide Steering Group of the OECD have been taken into account when relevant.

Results

The decision tree leads you from data and studies on the characteristics, identification and efficacy via application type and rate, an assessment or data on emission, exposure, effects to non-target organisms (NTO) and via the evaluation in which risk criteria are used, to a final decision whether the environmental risk to that particular NTO is acceptable or not. This is done by answering questions with yes or no, e.g., whether the exposure of NTOs exceeds background levels or whether the MCPP is infectious and/or pathogenic to one or more NTOs under the recommended application rate. If you want the figure of this tree, please contact the author. One should always assess whether data on the characterisation, identification, efficacy, emission, exposure, effects to non-target organisms — whether by toxicity, competitivety, allergenicity, infectivity, or pathogenicity — are acceptable and

useful for the safety evaluation. Unreliable data should not be used for the safety evaluation. A protocol to assess the reliability and usefulness of submitted data has been provided.

The decision tree stipulates the importance of the submitted data and information on characterisation, identification, efficacy and intended use. Respecting the effects assessment, the tree focuses on the toxic effects of an MCPP. The tree zooms in on the *potential* infectivity and pathogenicity, only if an MCPP is not toxic, allergenic or competitive to an NTO. If the MCPP is toxic, the safety evaluation proceeds in conformity with the EU evaluation of a chemical synthetic pesticide. Then a toxicity exposure ratio will be calculated, if possible, and the risk criteria for chemical synthetic pesticides will be used.

Discussion and conclusions

An environmental risk decision tree is proposed as a registration tool for MCPPs prior to their marketing (Mensink & Scheepmaker, 2007). This tree and the accompanying guidance document should enable risk assessors and environmental scientists to verify the risk criteria and descriptions respecting the potential environmental behaviour, fate and effects of an MCPP under review. In this way, it should be possible to discern whether a risk is acceptable or not. Case by case expert judgment, however, remains necessary in view of (a) the limited knowledge of modes of action, the role of toxins and enzymes, and the microbial population dynamics, (b) the limited experience of EU countries with regulatory test protocols for micro-organisms, (c) taxonomical difficulties in relation to the "indigenousness" of active micro-organisms, (d) long-term effects of MCPPs that are not well studied (d) difficulties in extrapolating laboratory data to the field.

An environmental safety evaluation without data is impossible. However, depending on the submitted data and the overall picture of the product's environmental safety that emerges, some data may be waived. Waivers should be properly substantiated, e.g., in case studies or data on relevant metabolites have not been submitted. Bridging and familiarity studies to support extrapolation of micro-organism strain A to strain B may be helpful. For mitosporic fungi, however, the use of such studies is limited as taxonomic issues may be under discussion. On the other hand, taxonomy based on molecular, biochemical or genetical characteristics is helpful as analytical and detection methods are under improvement and new combinations of DNA or RNA techniques are being developed. Taxonomy can be helpful to determine the 'indigenousness' of a species, strain or type. However, 'indigenousness' is a difficult concept and therefore of limited value.

Finally, it should be stated that in spite of *potential* infectivity, pathogenicity and toxicity there are no reports or studies indicating unacceptable risks of current MCPPs to NTOs. However, adverse effects due to active substances and coformulants should not be neglected as under particular conditions the effects of new MCPPs may be difficult to predict.

Reference

Mensink, BJWG; Scheepmaker JWA (2007). How to evaluate the environmental safety of microbial plant protection products: a proposal. Biocontrol Science and Technology **17**, 3-20.