Session 5A

New Compounds, New Concepts, New Uses and New Approaches 2

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Platform Papers:	5A-1 to 5A-7

Genetic technologies to enhance the Sterile Insect Technique

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Introduction

The Sterile Insect Technique (SIT) is an effective, species-specific and environmentally friendly method for controlling pest populations. It has been used successfully for control of several insect species in programmes targeting local eradication, suppression below economic thresholds, and prevention of establishment. Modern genetic methods hold out the prospect of significant operational and cost-effectiveness improvements to the SIT, and for extension of the SIT to a broader range of pest species.

Genetic improvements include: more reliable identification of released individuals, by providing a genetic marker allowing easy discrimination between wild type and released insects; removing the need for radiation-sterilisation, by providing 'genetic sterilisation'; reducing the hazard posed by non-irradiated accidental releases from the mass-rearing facility by arranging that the insects need an artificially-provided condition, for example a dietary supplement, in order to survive or reproduce; providing automated sex-separation prior to release to eliminate females from the release population ('genetic sexing'). Oxitec has demonstrated all of these properties in several insect strains including Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), pink bollworm (*Pectinophora gossypiella*) and the yellow fever mosquito (*Aedes aegypti*).

Genetic markers

In SIT, it is essential to be able to distinguish the released control agents (sterile insects) from the target wild population. Currently, this is done by adding a dye to food, or by dusting with fluorescent powder. Genetics could provide an alternative marking system, either by classical genetics, for example a visible mutation or by recombinant DNA methods (e.g. Alphey, 2002; Handler & Harrell, 2001; Horn *et al.*, 2002). Genes expressing fluorescent proteins have been incorporated into *Drosophila*, several tephritid fruit flies, moths and mosquitoes and it is clear that such markers could be provided in most species of interest for SIT.

The use of genetic markers may improve the speed and accuracy with which trap catches may be analyzed. In particular, since genetic markers can mark all tissues, and uncertain identifications can be confirmed by molecular analysis (e.g. PCR-based methods), their incorporation into a release strain should reduce the number of false positives (sterile insects mis-identified as wild type) and consequent quarantine responses. If fluorescent proteins are used to label sperm, it should be possible to determine whether a trapped female had mated a wild or a released male before she was captured, again avoiding unnecessary quarantine responses.

Genetic sterilisation

Current SIT programmes use ionizing radiation as the sterilizing principle. Irradiation induces random dominant lethal mutations in the gametes of irradiated insects. Progeny from such gametes therefore die, typically early in embryogenesis. However, irradiation has a significant negative impact on the performance of released insects in terms of their mating

competitiveness and lifespan and, therefore, on the cost and effectiveness of the programme, though the exact magnitude of this effect is still controversial.

Genetic systems have the potential to remove the need for irradiation. Oxitec has developed repressible dominant lethal genes into a system we refer to as RIDL®. (Alphey, 2002; Alphey & Andreasen, 2002; Gong *et al.*, 2005; Thomas *et al.*, 2000). In such a system, the lethal effect would be suppressed in the mass-rearing facility by a dietary supplement, effectively an antidote to the genetic system. RIDL insects could then be reared and released in the normal way, but without irradiation. Progeny of matings between RIDL insects and wild insects would inherit one copy of the RIDL construct. Lacking the antidote, they would die – much as if the released insects had been irradiated, but without the need to do so. Oxitec has generated genetically sterile strains of Mediterranean fruit fly, Mexican fruit fly, pink bollworm and *Aedes aegypti*.

By this approach, the financial cost and direct and indirect (e.g. handling) damage to the insects caused by the irradiation process may be eliminated. Significant additional benefit may be obtained by redesigning the mass-rearing and distribution process flow. Irradiation is only possible at late pupae or adult stage, and therefore dictates the current process. With RIDL, eggs or early pupae could be sent from the main rearing facility to cheaper local release stations, reducing cost and transit damage. It may be possible to release these life stages directly, saving the cost and damage incurred by the aerial release of fragile adults. RIDL will be applicable to insect pests where a sterilizing dose of radiation is too debilitating. For example, this opens up the field of mosquito control.

Genetic containment

SIT programmes currently rear fertile pest insects on a massive scale, which only become beneficial pest control agents once they have been sterilised with a sufficient dose of radiation. Large-scale release of non-irradiated insects due to a natural disaster or accident would be harmful at best, and potentially catastrophic. No such large-scale releases have yet occurred, though small non-irradiated releases have occasionally happened, for example of New World Screwworm in Mexico and Panama in 2003 (del Valle, 2003). The consequence of such an escape could be greatly mitigated by use of repressible lethal genetic systems such as those described above. RIDL strains cannot persist in the wild, as they or their progeny require the antidote to be present in their diet.

Use of such 'genetic containment' would seem to be a wise precaution in mass-rearing, particularly where the facility is located in a region where escape of the pest would be problematic, but also to reduce the negative impact of releasing even small numbers of inadequately irradiated insects in a control programme. Current mass-rearing facilities are often located far away from the sterile insect release areas, particularly when the goal is prevention of pest establishment. Transport of fragile sterile insects is expensive and damaging.

Rearing facilities have been moved from zones where pest eradication is complete at great expense, for example New World Screwworm production is being transferred from Mexico to Panama, where a new facility has been built at a cost of $40 million. Genetic containment would significantly reduce the risk of locating mass-rearing facilities close to their markets, improving the logistics costs of SIT programmes.

Genetic sexing

For many insects, it would be highly preferable to eliminate females from the release population. This is for two main reasons. First, the females of some species are damaging while the males are not. For example, sterile female fruit flies may damage fruit by oviposition (sting damage), while female mosquitoes may bite and transmit disease. Second, if males and females are released together, the males may court the released females, and consequently not seek out the wild females as effectively as if they had been released alone. This distraction effect has been shown to have a major impact on the male effectiveness of Mediterranean fruit fly in the field; in extensive field evaluation, males released on their own were estimated to be three to five times more effective than a similar number of males co-released with females (Rendón *et al.*, 2004). In many cases, females are not damaging, but do not contribute to pest control, and therefore represent unnecessary production and distribution costs.

Effective genetic sexing strains made by classical genetics are available for a few fruit fly species. In these strains, a chromosome translocation has moved a visible marker, such as pupal colour, onto the Y chromosome. In most cases, this is a recessive marker with a corresponding autosomal mutant or mutant plus deficiency (Hendrichs *et al.*, 1995). In the TSL strains developed by the IAEA group at Seibersdorf, Austria, both a pupal colour marker (*white pupa*) and a closely linked conditional lethal mutation (*temperature-sensitive-lethal*) are present, with a covering translocation for both on the Y chromosome. Thus, the females of the strain show a temperature-sensitive phenotype, but the males do not, so females can be eliminated by a suitable temperature treatment (Franz *et al.*, 1997; Robinson *et al.*, 1999). Unfortunately, the chromosome aberrations and mutations that are an integral part of these selection systems tend to reduce the overall performance of the flies that carry them, making them less effective agents for biological control. Despite much effort to minimize the problem, the translocations are also unstable; this instability is a significant problem when large populations of insects are reared and rearing methods need to be adapted to compensate. Furthermore, sexing strains made by classical genetics must be developed anew for each new species – genetic tools developed in one species by classical mutagenesis cannot be transferred to another species.

Recombinant DNA methods can provide alternative types of genetic sexing strains. Oxitec has developed a version of the RIDL system that is lethal only to female progeny in the absence of the antidote (Fu *et al.*, 2007). A female-specific strain of RIDL insects would be mass-reared in the presence of the antidote. The insects to be released would simply not be given the antidote – all the females would therefore automatically die, and the remaining RIDL males could be released without irradiation. They would mate with wild females; all the progeny from such a mating would inherit a copy of the RIDL system and so all the female progeny would die.

This is sufficient for population control, since the number of females essentially determines the reproductive potential of the population. Furthermore, the males survive and can pass the RIDL system on to at least some of the next generation. One design goal for these systems was that they should be relatively easy to transfer from one pest species to another – in contrast to classical systems – and this has indeed been our experience. Oxitec has generated genetic sexing strains for Mediterranean fruit fly, Mexican fruit fly and *Aedes aegypti*.

Biosafety and regulation

The use of genetically modified insects in SIT programmes will be subject to regulation. There are few conceivable hazards to people or the environment from use of RIDL technology in SIT applications. The USDA has put in place procedures for the regulation of transgenic arthropods and is preparing an environmental impact statement (EIS) review on the use of genetically engineered fruit flies and pink bollworm in plant pest control programmes. Approval for the first open release trial of a genetically modified insect was granted in 2006 for pink bollworm.

One specific technical issue about the environmental safety of transgenic insects has now been solved. This is the hypothetical possibility that the use of non-autonomous transposons as gene vectors might somehow destabilize the inserted transgenic construct, perhaps even, in the most improbable scenario, leading to movement of the construct to another species, so-called horizontal gene transfer (Alphey *et al.*, 2002; Handler, 2004; Hoy, 2000; Wimmer, 2003). However, recently methods have been developed that reduce (Handler *et al.*, 2004) or eliminate (Dafa'alla *et al.*, 2006) this hypothetical risk by eliminating one or both ends of the transposon, respectively.

Conclusion

Modern genetic methods are poised to make a series of significant improvements to the SIT. The combined effect should be to improve the cost-effectiveness of the SIT against a variety of pests of agricultural, veterinary and medical importance. This will extend the range of circumstances in which SIT is the preferred pest control strategy, and improve the economics of control for those species.

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Modern petroleum spray oils: do they kill insects by asphyxiation? Evidence for an alternative mode of action

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Testing of an nC24 petroleum spray oil (PSO) against the cotton aphid *Aphis gossypii* revealed signs of mortality inconsistent with anoxia, which is the most widely held theory about the mode of action of PSOs on insects. Treated insects died quickly with most of the mortality occurring within the first 10 minutes after spraying. The aphids became paralyzed and flaccid soon after contact with the oil and their cuticle became dehydrated and dark. Within a few hours of death, they had dehydrated completely (Najar-Rodriguez *et al.*, 2007). This rapid knock-down effect and the symptoms of the dying insects suggested a mode of action other than suffocation, which is a protracted process that may take hours in insects (deOng *et al.*, 1927). We, therefore, investigated the main mechanism(s) by which the PSO interacts with the insect tissues and ultimately kills them. The oil was also tested against *Spodoptera litura* caterpillars because they are not killed as quickly as aphids, so accurate determination of the course of symptom development and toxicology was easier. Confocal microscopy was used to determine the penetration of the PSO into the insects' bodies. The ability of the oil to penetrate insect cell membranes was also tested on an insect cell line *in vitro*. Further, the electrophysiological effect of the oil on nerve function was tested on isolated insect ganglia. This approach was extended to include amphibian and mammalian neuromuscular junctions, to establish at what level disruption of nerve function by the oil occurs.

The results demonstrate that the PSO tested in this study kills insects via a mode of action different from suffocation. Rapid penetration of the PSO through the insect cuticle is followed by accumulation of the oil in lipid-containing tissues, primarily the fat bodies and the central nervous system (CNS), and finally penetration into the nerve cells. Throughout the entire process, no signs of oil accumulation within the tracheae were observed. Thus, it seems unlikely that suffocation is taking place at any stage of the toxicity process. *In vitro* tests with isolated insect cells further confirmed that the oil is able to penetrate the cell cytoplasm and induce 100% mortality within two minutes of application. Electrophysiological studies revealed that oil accumulation in the nerve ganglia and in the peripheral nerves has a direct effect on suppressing synaptic transmission of insect nerves as well as frog and mouse neuromuscular junctions. This effect is reversible though, indicating that strong binding to receptors or to the active site of enzymes is not occurring. On intact neuromuscular junction preparations, the PSO reduced direct nerve stimulation by 100% and reduced direct muscle stimulation by 40%, indicating that the oil is preventing the passage of motor stimulation to the muscles. If high frequency stimulations (50 Hz) were used on the same preparations, nerve responses were recovered. The effects of the oil outlined above all indicate that the PSO is acting at the pre-synaptic level. Whether that effect is manifested in the membranes along the length of the axons or is confined to the

vicinity of the pre-synaptic membranes remains unclear. Whatever the mechanism though, the effect of the oil on synaptic transmission could cause all vital processes involving CNS coordination to cease. This effect seems to be reversible when the oil is withdrawn, at least within the experimental time frames used here. However, once the PSO is applied to insects in the field, it remains within them indefinitely, so the effect on the CNS could be thought of as being irreversible. Oil penetration of the nerve cells would thus be fatal to these cells and ultimately to the insects.

The findings of our study have important implications for the design of IPM strategies based on the PSO studied here. That the PSO affects nerve activity suggests the oil could be integrated into resistance management strategies for synthetic insecticides that target the CNS of insects (e.g. pyrethroids and organophosphates) and which kill in different ways to the oil. Furthermore, the PSO could be used as a means of increasing the penetration and toxicity of new botanical pesticides.

A direct toxic effect of a new commercial nC15 petroleum oil to the peripheral nerves of the lightbrown apple moth, *Epiphas postvittana* has also been recently demonstrated (Taverner *et al.*, 2001), thus indicating that the neurotoxic nature of modern PSOs extends to different types of oils, regardless of their carbon content. If this is the case, the alternative uses outlined above should apply also to most modern PSOs.

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A novel microencapsulated formulation of pyrethrins to control resistant pests

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Insecticide resistance can be caused by various factors, the most important of which are metabolic detoxification and target-site resistance. The enzymes which can confer metabolic resistance are esterases, glutathione-*S*-transferases and mixed function oxidases.

Synergists are natural or synthetic chemicals, which alone are non-toxic at the rate of treatment, but which significantly increase the toxicity of an insecticide against insect pests. Synergism thus occurs when the toxicity of a mixture is greater than expected on the basis of the independent toxicity of each of the components. This means, for example, that insecticides that are readily metabolised become more potent when applied with a synergist that blocks insecticide metabolism.

Piperonyl butoxide (PBO) was the first truly effective and commercially viable synergist to be developed. It was initially thought to be a specific inhibitor of microsomal oxidases, but more recently it has been shown to inhibit resistance associated-esterases (Gunning *et al.*, 1998; Young *et al.*, 2005; 2006). Because esterase inhibition by PBO does not occur immediately, but after several hours (Young *et al.*, 2005; 2006), application of PBO prior to treatment with the active insecticide gives optimal results for mortality. This is known as temporal synergism and can be achieved through the use of microencapsulated formulations.

Table 1 shows results with a new microencapsulated formulation containing PBO and natural pyrethrins which has the potential to give enhanced protection in the field. The formulation, which gives an initial burst of PBO followed by the slow release of pyrethrins, results in resistant insects being killed. Even a very resistant clone of *Myzus persicae* containing both metabolic and target-site resistance, can be controlled using the 'smart' formulation. This would be approved by the organic market in the UK since PBO is approved by the UK Soil Association. However, given that PBO is not an 'approved organic' in the rest of Europe, an alternative synergist would be required there.

A standard assay for screening putative synergists can be performed by measuring the synergist's ability to inhibit resistance-associated esterases *in vitro*. Esterase activity can be detected in a simple laboratory assay involving esters of 1-naphthol.

However, the standard biochemical assay to measure esterase inhibition by PBO has been found to be an unreliable indicator for several different insects, since *in vivo* studies have found synergism to be occurring (Table 1) even though inhibition cannot be detected *in vitro* (unpublished data). The use of a novel assay measuring AChE activity has now been developed which demonstrates the *in vitro* binding of the esterases by PBO and also enables putative synergists to be screened.

Table 1. Preliminary bioassay results for *Myzus persicae*, 794jz clone.

Method	Treatment	LC_{50} (ppm)	SE (±)	SR
Topical application	Pyrethrins	1142	143	1.0
	PBO/Pyrethrins (tank mix 4:1)	214	24	5.3
	PBO pre-treatment followed by pyrethrins	115	10	9.9
Leaf dip	Microencapsulated pyrethrins	>3000	nc	1.0
	PBO + microencapsulated pyrethrins	638	183	>4.7

LC_{50}: concentration of pyrethrins required for mortality in 50% of the population; SE: standard error; SR: synergistic ratio.

This new assay is now being used to screen for botanic extracts that show potential to be used as a synergist to pyrethrins. The work is in collaboration with Botanical Resources, Australia.

Unlike a standard spectrophotometric assay which directly measures esterase activity and the ability of a synergist to inhibit the esterases, the modified synergist assay indirectly measures the inhibition of esterases (E4 from *M. persicae*) by means of assaying the 'protective' effect provided by E4 to AChE. The AChE is inhibited by a potent AChE inhibitor, and the effect of pre-incubation of E4 or a synergist/E4 mix is compared. If the protective effect is lost, the synergist can be considered to have bound to the esterase.

It appears that when PBO binds to the esterases it does not always cover the active site, hence the inhibition by PBO cannot always be seen *in vitro*. Thus, different insects could have slightly different sized/shaped esterases. If the spatial distance between the two binding sites (active site and PBO-binding site) is small, the PBO would access its binding site on the esterase and block the substrate as a result. This explains why a standard esterase assay will reveal PBO inhibition of esterases from some insects, but not others. The reason that the PBO works well as a synergist *in vivo* is that the presence of PBO stops the esterase from binding to the insecticide regardless of the spatial positions of the two binding sites.

The novel approach to screen for putative synergists described in this paper enables large numbers of samples to be screened quickly. Successful synergists will be subsequently trialed in traditional bioassays to assess their effect *in vivo*.

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A summary of six years of greenhouse, microplot and field experimentation with a new in-furrow, at-planting material for the management of plant parasitic nematodes of major agricultural crops in the southern United States

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Introduction

Plant parasitic nematodes are a serious and insidious agricultural production constraint. They are microscopic, subtle pathogens that rarely make their presence known to the untrained eye. The phytopathological literature is replete with documentation of the national and international economic impact of crop losses caused by parasitic nematodes. The pathology that nematodes cause is often inaccurately attributed to factors such as plant nutritional and/or water deficiencies or excesses, soil-inhabiting fungi, bacteria and insects, or undesirable soil structure, fertility or topography. Across major agricultural regions and crops of the world, the annual loss caused by plant-parasitic nematodes is 10-20%, an estimate that translates into hundreds of millions of dollars. In regions of the world where there is great crop diversity and where winter conditions are relatively mild, losses certainly exceed these estimates.

In America, cotton, soybean, cereals and other major crops are parasitized by over 120 different species of nematodes. Vegetables and other more regional annual crops such as rice, sugarcane, and corn are each parasitized by a wide spectrum of ectoparasitic and endoparasitic nematode species. Additionally, perennial crops such as citrus, pecan, peach, and many forest-related plant species, especially *Pinus* spp., are also known hosts and susceptible to damage from many root, stem, and foliar-feeding nematode parasites.

There is a high degree of interactivity between nematodes and other plant pest species. Volumes of research data document 'plant pest complexes' in which resistance to one nematode species is lost in the presence of a second nematode species, or in which the presence of nematodes influences the pathogenicity and/or behavior of associated fungi, insects and weeds. Data from these studies clearly demonstrate that concomitant parasitism can influence both plant growth and pathogen/pest reproduction additively, synergistically or antagonistically. The latter, antagonism which results usually from physical crowding of the rhizosphere and/or the production of anti-helminthic compounds, is almost never employed intentionally as a means to maintain pest populations below damage threshold levels. Other documented types of pest suppression such as the use of allelochemicals, green-manure crops, cultural practices and solarization have historically been subordinated in favor of traditional, and for the most part environmentally-irresponsible, pesticides. Nematicides in particular produce undesirable ecological consequences and many are currently under close scrutiny or have been banned altogether in recent years.

The development of sustainable, profitable agricultural production systems must be linked to efficient, economical and environmentally responsible pest management strategies. Included in these is a new arsenal of environmentally responsible pesticides. One of this new generation of pesticides is a nematicide, Agri-Terra, produced by Cal-Agri Products, LLC of Los Angeles, California. In the USA, this material has been tested at leading universities in California, Florida, Idaho, Louisiana, Minnesota, North Carolina, New York

and Oregon. At Louisiana State University, greenhouse, microplot and field trials have been ongoing since 2000.

Materials and methods

The microplot is a controlled environment system in which the efficacy of an experimental nematicide can be evaluated without interference from the unknown and unmanaged biological and non-biological conditions inherent in field trials. Microplots employed in these studies were clay pots with top diameters of 30.5 or 80.4 cm, depths of 25.0 and 33.8 cm and soil capacities of 15 and 35 kg, respectively. Forty-eight microplots were spaced 1 m apart in a 6-by-14 pattern in each of seven research areas. Each area is bounded by a 17-m-long by 9-m-wide aluminum quonset hut skeletal frame open at both ends and covered with one layer of clear, 6-mm thick polyethylene greenhouse film and one layer of 20% reflective foilcloth. Light intensity under the reflective cloth was measured as $512\mu E \cdot s^{-1} \cdot m^{-2}$, which is approximately 78% of full sunlight. Each of these covered areas, which are necessary to protect plants in microplots from rainfall, is equipped with overhead fans and an automated irrigation system which allows for maintenance of near-natural air and soil temperatures and moisture conditions.

Soil used in microplots was a steam-sterilized Commerce silt loam (fine-silty, mixed, superactive, nonacid, Thermic Fluvaquentic Endoaquepts) with a pH of 6.8 and an organic matter content of approximately 1%. Crop species that have been employed in microplot trials include sugarcane, cotton, soybean, rice, tomato, bell pepper, cucumber, lettuce, cabbage, mustard green and endive. Cotton, soybean and rice seed were planted directly. Sugarcane was established from vegetative cuttings. Vegetables were grown from seed in the greenhouse and 20-24 day old seedlings were transplanted. Twenty-four hours prior to planting, soil in the microplots was infested with nematode species at preplant levels most commonly associated with the crop in Louisiana. At the time of planting, experimental treatments representing a range of concentrations of the material as well as appropriate phytotoxicity, pathogenicity and 'complete (no treatment)' controls were established. Microplots were fertilized twice monthly with Peter's Complete Nutrient Solution (8 ml/kg soil) and fungicides and insecticides were applied as warranted to manage foliar pest species.

The duration of all microplot trials was full-season and plant growth data were collected at harvest. On the day microplot trials were terminated, soil samples (400 g per microplot [6 cores from around the base of each plant, each core 2 cm wide × 20 cm deep]) were collected from each microplot immediately after excising plant tops and placing them into paper bags for drying at 43 C for 96 hours. Root balls were then removed from microplots and root systems were washed free of soil, inspected for damage and/or evaluated for degree of galling by root-knot nematode, photographed where appropriate, and placed into paper bags for drying along with plant tops. Soil samples were taken to the nematology laboratory and nematodes were extracted from a 250 g composite subsample using a common hand-sieving and centrifugal-sugar flotation technique. Following extraction of nematodes from soil, an inverted microscope was employed at a magnification of × 60 to enumerate and sort plant-parasitic nematodes according to genus and species.

Prior to establishment of field trials, soil samples from the test site were collected and submitted for analysis to the LSU Soil Testing Laboratory. Soil fertility and pH were then adjusted as per recommendations for the crop to be produced. During the growing season,

foliar pests and weeds were managed using pesticides recommended by the LSU Cooperative Extension Service Plant Disease Control Guide. A randomized complete block design was employed for all crops. Plots were 27.7 m long by 4 rows wide. There was a 3 m gap between replications and a 1 m gap between plots. Crops were established from seed, cuttings or transplants as described for microplot trials. Soil samples (20 cores [1.9 cm diameter by 15 cm deep] per plot) for analysis of nematode populations were collected from the root zone between rows 2 and 3 at planting and at harvest and handled as described earlier for microplot trials. With vegetables the material was applied as a fine mist spray to a 'V-Shaped' furrow in the soil and immediately covered with the 2 mm black plastic commonly used for commercial vegetable production. Vegetable seedlings were transplanted within 30 minutes of treatment establishment by punching holes in the plastic at transplant marking indicators on the plastic. A drip-irrigation line was also laid on the soil surface as the plastic was applied, but no supplemental irrigation was applied until 24 hours following the establishment of treatments. Data from all microplot and field trials were analyzed with the 'Fit Y by X' Module of JMP, the Macintosh equivalent of SAS (SAS Institute, Cary, NC) and Tukey's HSD (Honestly Significant Difference) test for main treatment effects. Unless indicated otherwise, differences noted were significant at the 5% level.

Results

Results of all research trials, field and microplot, are summarized in Table 1. Across crops, years and environments, significant plant growth responses were obtained with 7 of 11 crops and significant yield responses with 5 of 11. Moreover, significant control of indigenous nematode populations was observed in every single trial, with the single exception of the field trial with pepper in which the cultivar employed in the trial was not a host of the predominant nematode species, *Rotylenchulus reniformis*, the reniform nematode.

Table 1. Summary of six years of microplot and field trials.

Crop species used in trial	No. of years in Micro-plot (M)	No. of years in Field (F)	No. of years with significant growth response	No. of years with significant yield response	No. of years with significant nematode population control
Sugarcane	3	3	3M, 0F	3M, 1F	3M, 3F
Cotton	4	5	4M, 5F	4M, 5F	4M, 5F
Soybean	3	1	2M, 0F	0M, 0F	2M, 1F
Rice	3	0	1M	0M	3M
Tomato	4	1	2M, 0F	2M, 1F	4M, 1F
Bell pepper	3	1	3M, 0F	3M, 0F	3M, 0F
Cucumber	2	1	2M, 0F	2M, 1F	2M, 1F
Lettuce	1	0	0M	0M	1M
Cabbage	1	0	0M	1M	1M
Mustard green	1	0	0M	0M	1M
Endive	1	0	0M	0M	1M

Overwhelmingly, the data for cotton, tomato, cucumber and bell pepper were the most dramatic and consistent, although excellent nematode control was observed for all crops. The following Tables 2-6 present these data in much greater detail. (In all the Tables, * and ** indicate differences from the non-treated control that are significant at the 5% and 1% levels, respectively).

Table 2. Cotton (cvs. LA887 in 2000-2002 and Deltapine 434RR in 2003 and 2006) seed yield (kg/55.4 m) and reniform nematode soil population data from field plots treated or not treated with the new product

Year of trial	Product at 112.3 L/ha (10 g/A)	Non-treated control	Number of *R. reniformis*/500cc soil at harvest	
			treated plots	non-treated plots
2000	5.3*	2.5	5,008**	28,755
2001	6.7**	3.1	5,736**	23,202
2002	10.8**	5.2	5,248**	17,330
2003	3.7*	0.7	3,063**	19,751
2006	6.9**	2.1	7,855**	26,847
Average	6.7	2.7	5,382	23,177

Table 3. Cotton (cv. Deltapine 434RR) boll set, seed yield (g/plant), and nematode (*R. reniformis*) soil population data from microplots treated or not treated with the new product

Year of trial	Dry weight (g) at harvest			No. of bolls per plant	Seed cotton (g) per plant	No. of nematodes per microplot at harvest
	root	top	plant			
2002 (treated)	84.1*	229.3**	313.4*	33	52*	83,814**
2002 (non-treated)	67.3	170.7	238.0	26	31	250,657
2003 (treated)	76.8*	326.8**	403.6**	40**	60**	108,266*
2003 (non-treated)	59.0	147.3	209.3	19	37	195,004
2004 (treated)	90.5*	254.1*	344.6*	37*	51**	50,930**
2004 (non-treated)	73.3	179.0	252.3	24	26	277,420
2005 (treated)	70.2*	182.6	252.8*	31	Katrina	132,184**
2005 (non-treated)	53.4	133.6	187.0	22	Katrina	220,252
2005 (non-treated)	53.4	133.6	187.0	22	Katrina	220,252

Table 4. Tomato (cv. BHN 444) fruit yield (cumulative over the season) in 2006 field trial. Fruit was sized using an industry-standard template. Preplant reniform nematode soil densities averaged 2,827 per 500cc; and, harvest densities averaged 3,792 and 14,280 individuals in treated and non-treated plots, respectively.

Tomato fruit size category	Yield (kg) from plots treated with112.3 L/ha (10 g/A) of product	Yield (kg) from non-treated control plots
Max large	4.2	4.0
Extra large	21.1**	9.9
Large	45.9**	30.6
Medium	23.2	14.7
Small	0.6	2.1
Culls	16.3	14.6

Table 5. Cucumber (cv. Dasher II) fruit yield (cumulative over the season) in 2006 field trial. Fruit was sized using an industry-standard template. Preplant reniform nematode soil densities averaged 2,699 per 500cc; and, harvest densities averaged 6,908 and 24,592 individuals in treated and non-treated plots, respectively.

Cucumber fruit size category	Yield (kg) from plots treated with112.3 L/ha (10 g/A) of product	Yield (kg) from non-treated control plots
Super select	47.8*	35.9
Select	46.5**	26.7
Small-fancy	2.0	4.7
Large	9.8	11.8
Culls	22.9	26.5

Table 6. Bell pepper (cv. Keystone) fruit yields per plant and root-knot nematode (*Meloidogyne incognita*) soil population data averaged over 3 years (2003-005) in microplots Harvest intervals 1, 2, and 3 were 67-69, 74-77 and 93-96 days after transplanting, respectively.

Harvest Interval	Yield (kg) from microplots treated with112.3 L/ha (10 g/A) of product	Yield (kg) from non-treated control microplots	Number of *R. reniformis*/500cc soil at harvest	
			treated microplots	non-treated microplots
1	1.2**	0.4	476**	2,385
2	1.2*	0.8	no data	no data
3	1.1**	0.3	1,004**	6,907
Season total	3.5**	1.5	----------	----------

In each trial over 5 years, the yields of seed cotton (Table 2) were at least doubled in plots that received the 112.3 L/ha rate of this new material. Concomitantly, populations of the most serious pest of cotton, the reniform nematode, were reduced by an average of 77%. Since there is currently no commercial cultivar resistance to this nematode available, this material has the potential to impact cotton production in the south and elsewhere significantly. Microplot data (Table 3) for cotton also produced consistently positive plant responses and efficacy data. Weights of root and shoot systems were increased significantly by application of this product. Enhanced plant growth, usually visible as taller and more robust plants during the first 3 weeks following application, produced a significantly greater number of bolls per plant in 2 of the 4 trials and a significant increase in seed cotton per plant in 3 of the trials. The 2005 microplot trial was interrupted by hurricane Katrina and yield data were lost. Nematode data for all microplot trials produced efficacy results that paralleled those of field trials, with reductions in reniform populations averaging 62% over the four years.

The vegetable field trials (Tables 4-6), established on university property by policy since the new material is not yet federally labeled, were conducted in cooperation with LSU AgCenter horticulture faculty who supervised crop establishment, production and grading of fruit so that conditions simulated a commercial operation. The only exception to this protocol was that harvesting of crops was done by hand to ensure that all produce was harvested from each plot. Yields of tomato fruit in the extra large and large size categories were increased significantly over those from non-treated control plots. Treatments did not differ significantly for fruit in the max large, medium, small and cull categories. The new material also produced significant yield enhancement of cucumber fruit in the super select and select categories. Yields of cucumbers in other size categories were not influenced significantly by treatment. Nematode populations, which were primarily reniform nematode, were reduced significantly by treatment in both tomato and cucumber field plots (data not presented). Averaged over three years in the microplot environment, 2003-2005, yields of bell pepper fruit treated with the new material were increased significantly from 0.4 to 1.2 kg per plant at harvest 1, from 0.8 to 1.2 at harvest 2 and from 0.3 to 1.1 kg per plant at harvest 3. The total season fruit yield over the three years was 1.5 kg per plant for non-treated microplots and 3.5 kg per plant for treated microplots.

Turf project

In the spring and summer of 2006, trials were initiated to evaluate the effects of this material on nematodes associated with turf cultivars on golf greens in the Baton Rouge, LA area. Six local private golf courses (The University Club, The Bluffs, Carter Plantation, The Island, Le Triomphe, and Beaver Creek) participated in the project. After sampling established the presence of damaging populations and community levels of plant parasitic nematodes, (primarily stunt, lance, ring, stubby-root, sting and root-knot nematodes) greens were isolated from public traffic and aerated with a commercial aerating implement. Once aerated, a boom sprayer fitted with 8003 flat fan tips was employed to apply 0.9L of the material in 227.5L of water. After 14-21 days (Table 7.), pretreatment sites were re-sampled. Nematode community levels were reduced by 79% at The University Club, by 97% at The Bluffs, by 99% at Carter Plantation, by 76% at The Island, by 50% at Le Triomphe and by 93% at Beaver Creek. Within 90 days, there was marked improvement, both in turf density and color, at the two locations which had the highest pre-treatment nematode community levels. These studies continue at present at these and four other locations.

Table 7. Pre- and post-treatment nematode community levels associated with greens at six golf courses in the Baton Rouge, LA area

Golf course name	Pre-treatment nematode community density per 500cc of soil	Post-treatment nematode community density per 500cc of soil	Visible improvement in appearance of turf after 90 days
University Club	1,731**	359	No
The Bluffs	11,918**	250	Yes
Carter Plantation	9,469**	73	No
The Island	7,065**	1,803	No
Le Triomphe	6,212*	3,132	No
Beaver Creek	35,255**	2,371	Yes

What is the active ingredient and mechanism of action of this material?
Since this very promising material is not yet labeled for use in America, most formulation information will naturally remain proprietary until labeling requirements and data, currently under scrutiny by the American Environmental Protection Agency, are complete. This material is not a traditional nematicide. It is a surfactant-based mixture of seven components and its efficacy is a function of ingredient synergy. The mechanism of action of this product is almost certainly multi-faceted. Scanning electron microscope studies conducted in Spain with nematodes treated or not treated with the material show quite convincingly that most, but not all, treated nematodes showed a dermal coating and natural body openings (stylet orifice, excretory pore, phasmids as well as digestive and reproductive openings) that become 'plugged.' The coating and/or plugging of body openings would certainly inhibit or retard the proper exchange of gasses and fluids between the interior of the nematode and the external environment. With light microscopy, our laboratory has examined thousands of nematodes, representing many genera and all life stages including eggs that have been exposed to this material. While it is not uncommon for us to observe this effect, it is not apparent with all nematodes exposed to the material. These could quite possibly be compatible observations supporting a dermal coating theory due to the lack of detail available with a light microscope when compared with the electron microscope. Studies in our laboratory do, however, suggest quite strongly that the material inhibits the hatch of eggs of *R. reniformis* and the root-knot nematode, *Meloidogyne incognita* in soil.

The efficacy of this material has also been compared with that of Telone II at 45.3 and 90.6 kg ai/ha and Methyl Bromide at 130.9 kg ai/ha on cotton, soybean and sugarcane under field conditions. Both of these labeled materials produced significant reductions in soil populations of reniform and soybean cyst nematodes which ranged from 28-55% and yield increases of 8-30%. However, this new material produced reductions in nematode populations and increases in yield that were equal to those obtained with the two commercial but environmentally questionable materials (data are not presented here, but are published on pages 27-30 of the Proceedings of the Fifth International Conference on Alternatives to Methyl Bromide).

End notes

This material also has one additional property that is consistently apparent from inspection of data from the phytotoxicity, pathogenicity and 'complete (no treatment)' controls included in all microplot trials. Phytotoxicity control microplots receive the material, but are not infested with nematodes. Pathogenicity controls receive the nematodes, but no experimental material and complete controls receive neither nematodes nor experimental material. A comparison of plant growth data for the phytotoxicity and complete control treatments invariably shows a 6-10% greater value for phytotoxicity treatments over those of the complete control. While these values are almost always numerically higher, they are usually not significantly greater.

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Control of pineapple fusariosis with tannins and citrus extracts

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Introduction

Brazil is the biggest pineapple (*Ananas comosus*) producer in the world with high yields and excellent fruit quality. However, despite the development of technologies for the pineapple crop, huge losses still occur due to high incidences of fusariosis disease, a devastating fruit rot caused by the fungus *Fusarium subglutinans* which attacks not only the fruit, but also the whole plant and its slips which are used as propagating material in planting.

Traditionally, the control of this disease is based on preventive applications of chemical fungicides. Weekly applications of a mixture of benzimidazole fungicides and carbamate insecticides (to control a fruit pest which occasionally opens the way for the fungus) during the 4 to 5 week period in which the pineapple flowers are open efficiently control the fusariosis disease. On the other hand, there is increasing concern about the possible impact on the environment by fungicides and the onset of resistance to chemical crop protection agents together with a consumer move towards chemical free produce which has led to a search for agriculture products treated with natural substances.

The utilization of extracts of plants with antifungal properties is a promising ecological alternative to traditional protection promoted by the application of fungicides. It can also be associated with other integrated pest management practices contributing towards meeting a recent demand for organic products. Many species of medicinal plants contain phenols, quinones, saponins, flavonoids and terpenoids at a level high enough to repel insects and to prevent the occurrence of plant diseases. Phenolic compounds are of fundamental importance in the resistance of plants to the attack of fungi. Among important phenolic compounds in the resistance of plants to the attack of pathogens are flavonoids and tannins.

An alternative control program for pineapple fusariosis has been carried out since 1996 at the Pineapple Research Station in Northeastern Brazil in which the bark of a medicinal tree has been tested against the fungus *F. subglutinans* in laboratory and field experiments. This research has shown a significant reduction in the incidence of pineapple fusariosis from 31.2% (in the control treatment) to 7.6% with applications of aqueous extracts of the bark of a tree known as barbatiman (*Strypnodendron barbatiman*) instead of the application of chemical fungicides during the period in which the pineapple flowers were open.

However, despite the existence of many tree species rich in tannins in Brazilian rainforests, severe environmental regulations prohibit their utilization in agricultural IPM programs. Therefore, this research had the objective to study the effect of tannins from the cultivated black wattle tree (*Acacia mearnsii*) and of citrus extracts on the control of pineapple fusariosis.

Material and methods

This research was carried out at the Pineapple Research Station located in Sapé, Paraíba, in the Northeast of Brazil. The research station is located at an altitude of 124 m and sits over a luvisoil. Mean temperatures are around 30°C maximum and 21°C minimum with relative humidity varying from 70 to 100 %. In this region, the rainy season is from February to August with the highest rainfall in June and July.

The effect of sources and dosages of tannins on the growth of the fungus *F. subglutinans* in potato-dextrose-carragene medium was evaluated in complete randomized design laboratory experiments with 6 to 13 treatments and four replicates. Initially, home made (infusion) extracts of bark from two medicinal trees angico (*Anadenanthera colubrina*) and barbatiman were compared to extracts obtained by industrial processing of cultivated black wattle tree already available on the market as powdered products under many trade names (Weibull, Clarotan, Retan and Supertan with 72, 70, 74 and 70% of tanning substances, respectively). These are destined for the tannery and leather industries. Both powdered and infusion extracts were added to the culture media (giving a final concentration of 5% of each alternative product) just before being poured into 7 cm Petri dishes. These plates were inoculated with a 10 day old *F. subglutinans* colony and stored at room temperature for 7 days when the growth of the fungus was measured.

In addition, one of the black wattle powdered products was also tested at different concentrations (0; 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11 and 12%). These data were submitted to the analysis of variance using the F test for comparison of the treatments mean squares. The means of treatments were compared by the Tukey test at 5%.

A field experiment was carried out in a randomized block design with 16 treatments and 4 replicates during the raining season, which favors the disease. Each experimental unit was made of 70 pineapple plants pearl cultivar planted with 80 cm between rows and 30 cm within rows. In this experiment, tannins and citrus alternative products were applied as aqueous extracts during the flowering period in substitution to fungicides. Three sources of tannins were used: *A. colubrina* bark infusion (5 kg/100 L of water), *S. barbatiman* bark infusion (5 kg/100 L of water) and black wattle powdered bark (7.5 kg/100 L of water). Citrus extracts traded as Ecolife were used at a concentration of 500 ml/100 L of water, creolin disinfectants (1000 and 500 ml/100 L of water) and benomyl fungicide (75g a.i./ 100 L of water) were also used as comparisons.

A treatment containing only the insecticide carbaryl (150 g a.i./100 L of water) was included as a control. This same insecticide was applied with the other treatments in order to prevent the occurrence of insects which make holes in the fruit and open the way for the fungus which usually penetrates through the flowers apertures. All treated fruit were evaluated at harvest. Data referring to the number of attacked fruit by the fungus *F. subglutinans* were previously transformed in $\sqrt{(X+1)}$ and submitted to the analysis of variance using the F test for comparison of the treatments mean squares. The means of treatments were compared by Tukey's test at 5%.

Results and discussion

The addition of vegetable extracts rich in tannins to the culture medium resulted in a significant reduction of the mycelium growth of *F. subglutinans*. This was particularly true

with the commercial products that contained tannins of black wattle which produced a greater reduction than the home made extracts of medicinal trees (Table 1). This effect was directly proportional to the amount of product added to the culture media (Table 2).

Table 1. Effect of source of tannins on the growth of *F. subglutinans* in culture medium

Treatments:	Mycelium diameter after 7 days (cm)
1. Control	7.00 a
2. Clarotan black wattle tannin (5%)	2.16 d
3. Supertan black wattle tannin (5%)	2.18 d
4. Weibull black wattle tannin (5%)	1.75 d
5. Retan black wattle tannin (5%)	2.22 d
6. Angico (*A. colubrina*) tannin (5%)	3.47 c
7. Barbatimam (*S. barbatimam*) tannin (5%)	4.80 d
F test	260.78 **
CV (%)	8.63
lsd (Tukey at 5%)	0.52

Means followed by the same letter do not differ by the Tukey test at 5% probability

Table 2. Effect of dosages of black wattle (*A. mearnsii*) powdered product (with 72% tanning substances) on the growth of *F. subglutinans* in culture medium

Treatments:	Mycelium diameter after 7 days (cm)
1. Control	7.00 a
2. Black wattle tannin (1%)	4.80 b
3. Black wattle tannin (2%)	3.98 c
4. Black wattle tannin (3%)	3.47 d
5. Black wattle tannin (4%)	2.88 e
6. Black wattle tannin (5%)	2.25 f
7. Black wattle tannin (6%)	2.15 f
8. Black wattle tannin (7%)	1.55 g
9. Black wattle tannin (8%)	1.91 f
10. Black wattle tannin (9%)	1.55 g
11. Black wattle tannin (10%)	1.45 g
12. Black wattle tannin (11%)	1.35 g
13. Black wattle tannin (12%)	1.36 g
F test	415.60 **
CV(%)	6.86
lsd (Tukey at 5%)	0.37

Means followed by the same letter do not differ by the Tukey test at 5% probability

The efficiency of vegetable extracts against *F. subglutinans* was also evident in the field experiments (Table 3). The significant reduction in the incidence of pineapple fusariosis from more than 25% in the control treatment to less than 7% in the treatment with citrus extracts (rich in phytoalexins) and to less than 4% in the treatment with tannins of black wattle was due to the fact that those substances are either natural antibiotics or because they actively take part in biochemical processes which result in the strengthening of plant

defenses against fungi. The main mechanisms of antimicrobial action of tannins are based on their capacity of inhibiting enzymes, of modifying cellular metabolism by acting on membranes and of forming complexes with metal ions with their consequent reduced availability for microbial metabolism (Haslan, 1996).

Table 3. Incidence of pineapple fusariosis by treatments in field experiments

Treatments:	Number of attacked fruit [1]	Incidence
1. Absolute control	3.56 a	(25.77%)
2. Relative control (with carbaryl only)	2.93 ab	(16.53%)
3. Creolin disinfectant (1000 ml/100 L)	2.29 ab	(10.10%)
4. Creolin disinfectant (500 ml/100 L)	3.17 ab	(20.09%)
5. Citrus extract (applied once a week)	2.51 ab	(11.66%)
6. Citrus extract (applied twice a week)	1.77 ab	(6.52%)
7. *S. barbatiman* (applied once a week)	2.10 ab	(9.73%)
8. *S. barbatiman* (applied twice a week)	2.22 ab	(8.42%)
9. *A. colubrina* (applied once a week)	2.74 ab	(15.65%)
10. *A. colubrina* (applied twice a week)	2.41 ab	(12.11%)
11. Clarotan tannin (applied once a week)	1.60 b	(3.32%)
12. Clarotan tannin (applied twice a week)	1.54 b	(3.95%)
13. Weibull tannin (applied once a week)	1.86 ab	(9.43%)
14. Weibull tannin (applied twice a week)	2.23 ab	(8.62%)
15. Benomyl (applied once a week)	2.01 ab	(9.68%)
16. Benomyl (applied every other week)	1.99 ab	(6.73%)
F test	2.16 *	
CV(%)	32.12	
lsd (Tukey at 5%)	1.92	

Means followed by the same letter do not differ by the Tukey test at 5% probability

[1] Data transformed in $\sqrt{(X+1)}$

Conclusion

This research has shown that an environment friendly alternative control of pineapple fusariosis disease can be achieved through the use of vegetable extracts rich in tannins and vitamins which are natural plant defenses.

Reference

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Characterization of a novel carbendazim tolerant *Bacillus subtilis* strain with multiple plant growth promoting activities

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Abstract
We have isolated and characterized a novel strain of *Bacillus subtilis* MTCC 8141 that possesses the capability for: production of growth stimulating hormones; solubilization of phosphates; siderophore production; antibiotic production; and nitrogen fixation. In addition to these traits, the isolate showed strong *in vitro* inhibition (100% in mixed culture) of *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *lycopersici*. This is the first example of a biocontrol agent isolated from a tomato plant rhizosphere to exhibit multiple plant growth promoting activities. The ability to perform multifarious activities in tandem suggested the uniqueness of the isolate MTCC 8141. Furthermore, the isolate was able to tolerate concentration up to 2000 µg/ml carbendazim and 550 µg/ml streptomycin. Thus, the innate capability of the novel isolate for parallel plant growth promotion and high tolerance to carbendazim has significance in the integrated management of soil borne fungal diseases of tomato.

Introduction

Plant growth promoting rhizobacteria (PGPR) have gained world wide importance and interest because of their agricultural benefits and are thus potential tools for sustainable agriculture for the future. Although PGPR have been reported to influence plant growth, yield and nutrient uptake by a diverse array of mechanisms, the specific traits by which PGPR promote growth have been limited to the expression of one or more of the traits expressed at a given site of plant/microbe interaction. The potential benefit in the application of a single strain PGPR formulation has been demonstrated. The reported inconsistent performances and comparatively reduced beneficial effects in use might be due primarily to the expression of fewer plant growth promoting traits. Multiple strain mixtures of PGPR, each with a specific capability and function, may have certain advantages: broad spectrum activity; enhanced efficacy; and reliability of the biocontrol agent, thereby allowing the combination of various traits without the use of genetic engineering. The concept of using PGPR mixtures for disease control and plant growth promotion has already been well demonstrated in several crops.

However, in some instances root colonization of one member of a mixture can be severely affected. PGPR root colonization is an essential process in plant microbe interactions determining the optimum population level of the inoculated strain. All PGPR strains of a particular genus and species may not have identical metabolic capabilities and interactions with plants. Compatibility of individual strains in mixture among themselves, and with commonly used agrochemicals are also some of the constraints in the use of multiple strain mixtures of PGPR. Isolation of a PGPR strain with multiple plant growth promoting activities might help to address these problems. Recently such a PGPR strain of *Burkholderia* sp. has been isolated from the root nodule of *Mimosa pudica* (Pandey *et al.*, 2005). Research in our group focuses on the development of a formulation of a single PGPR strain with multiple plant growth promoting activities.

Greenhouse and field studies have demonstrated that treatment of seeds/seedlings of tomato with single or multiple strain preparations of PGPR has led to disease reduction and promotion of plant growth. PGPR has not yet been reported for tomato crops of the mid-hill temperate region of Himachal Pradesh, India, where tomato is the most remunerative cash crop. Thus, the aim of this study is to isolate biocontrol PGPR with multiple plant growth promoting activities.

Materials and methods

Isolation of PGPR

Replica plating technique originally developed to isolate auxotrophic mutants was used to isolate PGPR with multiple plant growth promoting activities. The tomato plants were carefully uprooted and up to 1.0 g of rhizosphere soil and root samples were used. The serially diluted suspension of soil/roots was spread on enriched soil extract medium. The isolated colonies that developed on the enriched medium (master plate) were replica plated onto the selective media: nitrogen free medium (Jensen, 1942) for N_2-fixing ability, Pikovskaya medium (Pikovskaya, 1948) for phosphate-solubilizing ability and chrome azurol S medium (Schwyn & Neilands, 1987) for siderophore production. All colonies were transferred to the same position as on master plate. At the end of incubation period, the number and location of colonies appearing on each plate were compared.

***In vitro* antagonism** - A dual plate method was used for *in vitro* screening of biocontrol PGPR. Per cent growth inhibition was calculated according to Vincent (1947) using the formula:

$$I = \frac{C\text{-}T}{C} \times 100$$

Where, I = per cent growth inhibition; C = growth in control; T = growth in treatment.

Phosphorus solubilization

Estimation of phosphorus was done in minimal medium amended with 5.0 g/l tricalcium phosphate (Sundara Rao & Sinha, 1963).

Siderophore production

The CAS assay (Schwyn & Neilands, 1987) was used to detect siderophore production in mineral salt medium with different concentration of Fe^{3+}. Positive reactions were recorded by a change in the colour of the CAS reagent from blue to orange or pink.

Indole acetic acid (IAA) production

Minimal medium supplemented with 500 µg/ml L-tryptophan was used for determination of IAA (Glick, 1995).

Production of antifungal antibiotic activity

The inhibitory effect of culture filtrate was determined using the poisoned food technique (Nene & Thapliyal, 1997). The inhibitory effect of cell pellet was determined using a dilution technique (Warnock, 1989).

Results and discussion

Isolation and selection of biocontrol PGPR

The conventional procedures employed for isolation and screening of PGPR strains for multiple plant growth promoting activity are both time consuming and limited in that they only select, bacteria for one type of biological trait. In brief, this entailed isolating a large

number of bacterial strains (often hundreds or even thousands) from rhizosphere soil/roots. These bacterial isolates were then tested for various plant growth promoting activities on different selective media.

In this study, the task of transferring several hundred colonies to test isolates with multiple functions is simplified by the use of a one step replica plating technique. The most predominant rhizobacteria were initially isolated and enumerated on enriched soil extract medium that supported the growth of many soil micro-organisms. After determining the location of colonies with multiple activities on replica plates on selective media and comparing with master plate, the organisms with multiple plant growth promoting activities were isolated. The procedure involved the replica plating of the colonies developed on rich and complete medium onto selective medium. The limited number of colonies on the selective media plates was screened for biocontrol activity against one or more fungal pathogens. The advantage of this method is that it enables the selection of biocontrol PGPR that promote plant growth directly (biofertilizers) as well as indirectly (biopesticides).

During the initial screening process using replica plating techniques, nineteen isolates grew on Pikovskaya medium, twelve on N_2 free medium and seven on chrome azurol S medium. Ten and nine isolates were antagonistic to *Fusarium oxysporum* and *Rhizoctonia solani*, respectively. Most of the isolates exhibited two or more than two common traits. However, concurrent production of siderophores, phosphorus solubilization, growth on N_2 free medium, antagonistic activity were exhibited by only three isolates. The strain SH-2 was specifically chosen based on its relatively higher antifungal and plant growth promoting activities.

In the dual culture technique, SH-2 showed more than 80% mycelial growth inhibition of both *F. oxysporum* and *R. solani*. A clear zone of inhibition was also observed. Of special interest was the discolouration of the mycelium even though there was no physical contact between the fungal mycelial and bacterium. The mixed culture experiment revealed complete inhibition (100%) of fungal growth.

On the basis of morphological and biochemical characteristics, the isolate SH-2 was presumptively identified as *Bacillus subtilis* MTCC 8141. The identification was confirmed from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology Chandigarh, India.

Some of the important properties of strain MTCC 8141 are depicted in Table 1. The characteristic features of strain MTCC 8141 are its ability to grow at wide range of temperature and pH, as well as to perform multifarious plant growth promoting activities in tandem.

The ability of the strain MTCC 8141 to grow at 550 µg/ml streptomycin and 2000 µg/ml carbendazim may now help to study its population dynamics in soil and plant roots. Pesticides in general and fungicides in particular are essential for disease management and in some cases they may show toxicity towards the introduced PGPR. The strain MTCC 8141 can be a useful component of integrated management of soil borne fungal diseases of tomato because of its high tolerance to carbendazim. The common mechanisms of plant growth promotion are: production of growth stimulating phytohormones; mobilization of phosphorus; siderophore production; antibiotic production; and nitrogen fixation.

Our isolate, *B. subtilis* MTCC 8141 possesses the capability to perform all these functions and thereby explaining its plant growth promoting potential. These factors were further investigated.

Table 1. Physiological properties of *Bacillus subtilis* MTCC 8141

Temperature (°C)	Growth	pH	Growth	NaCl (%)	Growth
25	Positive	5.0	Positive	2	Positive
35	Positive	6.0	Positive	4	Positive
40	Positive	7.0*	Positive	5	Positive
50*	Positive	8.0	Positive	7	Positive
55	Positive	9.0	Positive	8	Positive
		10.0	Positive	10	Positive

Plant growth promoting activities

Phosphates solubilization	Phosphate solubilization index (PSI) = 2.16
Nitrogen fixation	Positive
IAA production	Positive
Siderophore production	Positive (44.4% SU)
HCN production	Negative
Antagonistic activity:	
Fusarium oxysporium	Positive
Rhizoctonia solani	Positive
Sclerotinia sclerotorium	Positive
Dematophora necatrix	Positive

* Optimum temperature and pH for growth
PSI: clear zone diameter/growth diameter

Production of antibiotic activity

The strain MTCC 8141 showed antagonism against *R. solani* and *F. oxysporum* owing to the production of antimicrobial metabolites in the dual culture on solid agar medium. However, activity in culture filtrate was not detected. The activity was found to be associated with the cell pellet only. These results do not explain the mechanisms of antifungal metabolite production in the solid medium, but not in the liquid medium. The literature indicates (Sadfi *et al.*, 2002) that antibiotic production in such cases is induced by the presence of the fungal host. The ability of this strain to exhibit antifungal activity over a wide temperature (20-50°C) and pH (5.0-8.0) range reveals its usefulness under fluctuating environmental conditions.

IAA production

Strain MTCC 8141 grown in mineral salt medium with L-tryptophan exhibited a substantial production of IAA (Table 2). The production of IAA increased with time of incubation. 9.0 µg/ml of IAA was released in culture medium after 48 h incubation. Further, incubation did not increase the IAA production substantially. IAA production by microbes promotes root growth by directly stimulating plant cell elongation or cell-division (Glick & Panrose, 1998).

Table 2. Growth and IAA production by *B. subtilis* MTCC 8141.
Each value is the mean of triplicates

Incubation period (h)	Growth (log CFU/ml)	IAA production (µg/ml)
0	5.95	0.00
24	6.43	4.00
48	6.91	9.00
72	6.93	9.50
96	6.89	9.40

Phosphorus solubilization

Quantitative estimation of phosphorus solubilization revealed that a maximum of 272.4 mg/L of phosphorus was observed after 72 h of incubation (Table 3). Further incubation did not improve the level of solubilization. At the end of each day, the final pH was determined to find out whether the solubilization of phosphate was accompanied by the production of acids. Strain MTCC 8141 decreased the pH of the medium from neutral to 4.66 after 72 h of incubation. Thereafter, the pH of the medium remained stable. The decrease in pH clearly indicates the production of acids, which is considered to be responsible for P-solubilization (Pareek & Gaur, 1973).

Table 3. Growth and phosphorus solubilization *B. subtilis* MTCC 8141.
Each value is the mean of triplicates

Incubation period (h)	Growth (log CFU/ml)	Final pH	Phosphate solubilization (mg/L)
0	6.18	6.86	0.00
24	6.56	5.93	97.70
48	6.81	4.87	197.70
72	6.77	4.66	272.40
96	6.62	4.67	265.00

Conclusion

This study elucidates the mulitiple role of *Bacillus subtilis* strain MTCC 8141 and its wide action spectrum and plant growth promoting potential. Because of its high tolerance of carbendazim, this isolate could serve as a very effective biocontol PGPR inoculant in the integrated management of soil-borne diseases of tomato. The choice of such bacteria can further augment their utility as bio-inoculations in sustainable organic farming.

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Bacillus subtilis, strain QST713 bio-fungicide: pre-harvest applications for post-harvest disease control in fruit crops

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Abstract
Bacillus subtilis QST713, a naturally occurring bacterial isolate, was discovered and commercialized by AgraQuest, Inc., USA for foliar disease control in fruits and vegetables. It was approved by the US-EPA as a foliar fungicide in 2000 and is currently registered in more than 20 countries under the trade name, Serenade®. Studies utilizing pre-harvest applications of *B. subtilis* QST713 have documented efficacy against the post-harvest diseases in blueberries, stone fruit and grapes. *B. subtilis* QST 713 can be applied up to and including the day of harvest because there are no synthetic chemical residues. Activity against causal organisms of key post-harvest diseases in addition to a zero-day pre-harvest interval have demonstrated potential for *B. subtilis* QST 713 as a component of post-harvest disease control programs.

Introduction

The availability of year-round, high quality fruit and vegetables has been made possible by advances in long-term storage technologies, ethylene management and the use of synthetic fungicides for post-harvest disease control during shipment and storage. However, loss of synthetic fungicides due to the development of pathogen resistance, regulatory actions and increasing consumer demands for pesticide-free fruit and vegetables has created the need for new approaches to post-harvest disease control.

Microbial antagonists based on bacteria and yeast have demonstrated potential as alternatives to synthetic fungicides for post-harvest disease control when used in conjunction with an integrated approach involving all phases of production. *In vitro* activity against key post-harvest pathogens together with a zero-day pre-harvest interval formed the basis for studies designed to assess the potential of pre-harvest applications of *B. subtilis* QST713 for post-harvest disease control.

Blueberries

Studies have identified several important pathogens which contribute to post-harvest fruit rot in blueberries, one of the most important being *Alternaria tenuissima*. Current post-harvest treatments include SO_2 fumigation of harvested fruit prior to packing and shipment. Synthetic fungicides have also demonstrated efficacy, however their use is restricted by exporters and retailers due to the presence of chemical residues on treated fruit. Therefore, a study was conducted to evaluate pre-harvest applications of *B. subtilis* QST713 for post-harvest disease control in blueberries.

Methodology

A 0.3% w/w solution of *B. subtilis* QST713 wettable powder (WP) was applied with a backpack sprayer to 10 blueberry bushes, var. O'Neal, at fruit maturity. Water was also applied to 10 bushes as the untreated control. For both the QST713 treatment and the watercontrol, 400 berries were randomly selected 2 hours after application, placed in commercial packaging and stored at 0 °C. Fruit, packaged and stored under the same

conditions, was also collected from a single SO_2 fumigation treatment as a commercial standard comparison. Evaluation intervals consisted of 10, 20, 30 and 40 days after application and sample collection. One hundred berries for each treatment at each evaluation interval were placed in a humid chamber at ambient temperature and assessed for incidence of fruit infected with *Alternaria tenuissima* after 7 days. Data were statistically analyzed using ANOVA method and the DGC test with a probability of 0.05%.

Results

A pre-harvest application of *B. subtilis* QST713 provided significant efficacy against post-harvest *Alternaria tenuissima* infections on blueberries (Table 1). *B. subtilis* performance was statistically superior to the water control and SO_2 fumigation standard at all evaluation intervals. The results indicate that *B. subtilis* QST 713, applied in the field prior to harvest, is a potential tool for post-harvest disease control programs in blueberries.

Table 1. Efficacy of *B. subtilis* QST713 against post-harvest infections of blueberries

	% Infected fruit / days after harvest (*A. tenuissima*)			
Treatment	10	20	30	40
Water control	36 a	31 a	66 a	66 a
SO_2 fumigation	28 b	29 a	45 b	36 b
B. subtilus QST 713	18 c	11 b	27 c	16 c

Peaches

Stone fruit are extremely vulnerable to losses from post-harvest diseases, the most common of which is brown rot caused by the fungus *Monolinia fruticola*. A field trial was conducted to evaluate a pre-harvest application of *B. subtilis* QST713 for potential in controlling post-harvest brown rot infections on peaches.

Methodology

The aqueous suspension (AS) and wettable powder (WP) formulations of *B. subtilis* QST713 were applied to freestone peach trees, var. Faye Elberta, at 9.35 liters and 2.24 kg/ha respectively, on the day of harvest. For comparison, the standard synthetic fungicide tebuconazole, was applied at 280 g/ha. All treatments were applied with a handgun sprayer at 935 liters/ha. After the spray solution dried, disease-free fruit was randomly harvested from each treatment, placed in commercial packages, inoculated with *M. fruticola* spores and stored at room temperature. Evaluation of fruit infected by *M. fruticola* was conducted at 2, 3, 4, 5, 6 and 7 days after harvest. Diseased fruit counts were combined with previous counts so that analysis was conducted on cumulative infected fruit. Data were statistically analyzed utilizing the ANOVA method with a probability of 0.05%.

Results

All treatments provided significant control of brown rot at 2 and 3 days after harvest compared to the untreated control (Table 2).

At 4 days, only the AS formulation of *B. subtilis* QST 713 and the commercial standard tebuconazole provided a statistically significant reduction in infected fruit. Under the severe conditions of this trial, none of the treatments provided brown rot control at 5, 6 or 7 days after harvest, stored at room temperature.

Table 2. Efficacy of *B. subtilis* QST713 against brown rot of peaches

Treatment		Cumulative No. of infected fruit / days after harvest					
		2	3	4	5	6	7
B. subtilis	WP	2.5 b	7.8 b	11.0 ab	12.8 a	14.8 a	15.0 a
B. subtilis	AS	3.0 b	5.8 b	8.3 b	10.5 a	12.3 a	12.8 a
Tebuconazole		2.5 b	5.5 b	7.5 b	9.3 a	11.5 a	13.0 a
Untreated		6.3 a	11.5 a	14.3 a	15.0 a	16.8 a	17.0 a

Grapes

The most important fungus causing post-harvest rot of table grapes is *Botrytis cinerea*. This fungus can grow at temperatures as low as 0 °C and therefore, has potential to cause significant reductions in quality during long term, refrigerated storage and shipment. SO_2 fumigation prior to storage or generated by pads placed in shipping boxes are the most commonly used techniques used to control *B. cinerea* infections. However, SO_2 fumigation can cause injury and discoloration to berries depending upon the grape variety and treatment conditions. Additional concerns include the potential for allergic reactions from workers and consumers to the resulting sulfite residues. A semi-commercial trial assessing a pre-harvest application of *B. subtilis* QST713 for effects on quality variables and efficacy against post-harvest botrytis infections of table grapes stored under low temperature conditions was conducted.

Methodology

B. subtilis QST 713 wettable powder was applied to table grapes, var. Red Globe, at 1 and 2 kg/ha with commercial air-blast equipment at 1000 liters/ha. Fruit samples were randomly harvested from each treatment one day after application, placed in commercial packaging and stored at 0 °C, 90-95% RH. Fruit, sampled and stored under the same conditions, were packaged to include a 4.5 kg SO_2 fumigation generator as a commercial standard comparison. An untreated control was not included as this was a large-block trial conducted in a commercial vineyard. Evaluation intervals consisted of 0, 30, 60 and 90 days after sample collection. Fruit, at each evaluation interval, were placed in a refrigerator at 6-7 °C for 2 days after which evaluations for several quality parameters and counts of botrytis rotted berries were carried out. Data were statistically analyzed utilizing the Fisher LSD method with a probability of 0.05%.

Results

Both *B. subtilis* QST713 treatments provided post-harvest efficacy against botrytis rot comparable to the commercial SO_2 treatment at all evaluation periods (Table 3). With regard to percent weight loss, *B. subtilis* QST 713 treatments had statistically significant higher cluster weight losses compared to SO_2 at each evaluation however; all treatments had minimal cluster weight loss, less than 5% up to and including 90 days.

The SO_2 treatment also had less rachis browning at 30 days, but no difference in browning was observed between treatments at the 60 and 90 day evaluations. Botrytis rot control provided by a pre-harvest application of *B. subtilis* QST713 was equivalent to the SO_2 standard although the SO_2 treatment was slightly superior to *B. subtilis* QST713 for the quality parameters, weight loss and rachis browning.

Table 3. Efficacy of *B. subtilis* QST713 against post-harvest infections of grapes

Evaluation interval		Cluster			Botrytis rot
Days	Treatment	% Weight loss	% Removed berries	% Rachis browning	# Infected berries
30	SO_2	0.23 c	0 a	16 b	1 b
	B.s. 1 kg/ha	0.84 b	0 a	50 ab	2 a
	B.s. 2 kg/ha	1.73 a	0 a	84 a	2 ab
60	SO_2	0.69 c	0 a	34 a	1 b
	B.s. 1 kg/ha	1.72 b	1 a	34 a	6 a
	B.s. 2 kg/ha	3.10 a	0 a	50 a	2 b
90	SO_2	1.17 c	1 a	100 a	5 a
	B.s. 1 kg/ha	2.73 b	2 a	100 a	15 a
	B.s. 2 kg/ha	4.32 a	2 a	100 a	11 a

Conclusion

There is clearly a need for new post-harvest disease control technologies that address pathogen resistance, worker and consumer safety and environmental issues. The intense disease pressure associated with conditions in packing facilities has contributed to rapid development of resistance to synthetic fungicides. Additionally, there is an increasing demand from consumers and retailers for pesticide-free fruit and vegetables.

Results from these initial studies characterizing *B. subtilis* QST713 for efficacy against post-harvest diseases have shown that it represents a promising alternative to traditional treatments. Efficacy against causal organisms of key post-harvest diseases in blueberries, stone fruit and grapes using a pre-harvest application was demonstrated for *B. subtilis* QST713 under various storage conditions. Additional studies will be conducted to evaluate fully the potential of *B. subtilis* QST713 as a new tool for post-harvest disease control programs in fruit and vegetables.