ANTAGONISTIC POTENTIAL OF BACTERIAL ISOLATES ASSOCIATED WITH ENTOMOPATHOGENIC NEMATODES AGAINST TOMATO WILT CAUSED BY *FUSARIUM OXYSPORUM* F.SP., *LYCOPERSICI* UNDER GREENHOUSE CONDITIONS

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Abstract

Three concentrations of *Xenorhabdus nematophila* and *Xenorhabdus* spp., (4×10^5, 4×10^6, 4×10^7 cells/ml) were evaluated in the laboratory and in pot experiments to test their antagonistic effects on *Fusarium oxysporum* f.sp., *lycopersici*. All concentrations effectively inhibited its growth on agar plates. In soil under greenhouse conditions treatments with each bacterium at 4×10^7 cells/ml reduced the disease incidence of tomato by up to 40.38 and 47.54% respectively and there were significant increases of plant biomass by 198 and 211% respectively. The rhizosphere population of *Fusarium oxysporum* f.sp., *lycopersici* was reduced by 97%. The *Xenorhabdus* spp., was comparatively more effective than *X. nematophila*.

Introduction

Dutky *et al.*, (1964) suggested that the bacteria which live as symbionts of the entomopathogenic nematode *Steinernema carpocapsae* produce antibiotics which was confirmed by Akhurst (1982) who showed that two of these bacteria viz., *Xenorhabdus* spp., and *Photorhabdus luminescens* produce antibiotics that inhibit the growth of many bacteria and yeast species. Chen *et al.*, (1994) demonstrated the effect of *Xenorhabdus* and *Photorhabdus* on growth of some pathogenic and non pathogenic fungi *In vitro* and Vagelas *et al.*, (2004) achieved suppression of *Fusarium oxysporum* f.sp., *lycopersici* with the bacterium *Flavimonas oryzihabitans* in pot experiments. In this paper *Xenorhabdus* spp., that are found in species of *Steinernema* were evaluated as possible biocontrol agents against the root-infecting fungus, *Fusarium oxysporum* f.sp., *lycopersici,* the cause of wilt disease on tomato.

Materials and Methods

Isolation of the bacterial symbionts: Cadavers of greater wax moth larvae, *Galleria mellonella*, infected with *Steinernema carpocapsae* and *S. riobrave* were surface sterilized in 70% alcohol for 2 minutes, transferred to clean tissue to dry in a laminar airflow cabinet for 3 min, opened with sterile needles and a drop of haemolymph was streaked on to nutrient agar in 9 cm Petri-dishes and incubated at 28˚C. After 48 h single colonies of the bacteria *Xenorhabdus nematophila* and *Xenorhabdus* spp., from cadavers infected with *S. carpocapsae* and *S. riobrave* respectively were inoculated on nutrient agar plates and sub-cultured continually until colonies of uniform size and morphology were obtained. The pathogenicity of isolates was confirmed by injecting cells of the bacteria into *G. mellonella* larvae.

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Production of bacterial cells: A colony was inoculated into nutrient broth (18 g nutrient broth in 500 ml distilled water) in a flask and placed in a shaking incubator at 150 revolution/min for 24 h at 28˚C. The concentration of bacterial cells in the broth suspension was determined on a spectrophotometer at 600 nm wavelength.

Inhibition of fungal growth by *Xenorhabdus nematophilus* and *Xenorhabdus* spp.: A 1 ml suspension of *F. oxysporum* f.sp., *lycopersici* spores (10^6/ml) isolated from wilt disease of tomato was added to 25ml of cooling nutrient agar in 9 cm Petri dishes. When solid, a 5 mm plug was removed from the centre of the dish and to this hole was added 0.5 ml of nutrient broth (NB) containing $4x10^5$, $4x10^6$ or $4x10^7$ cells/ml of *Xenorhabdus nematophila* and *Xenorhabdus* spp., control dishes had NB only. All dishes were sealed and incubated for 6 days at 28ºC. Observations of the inhibition were recorded after 72h and where appropriate after 6 days.

Fungal pathogen: *Fusarium oxysporum* f.sp*., lycopersici* (*Fol*) originally obtained from CABI-Bioscience, Egham (IMI 194417) was cultured on PDA plates for 7 to 10 days at 28 ± 2ºC until sporulation. The pathogenicity test was performed. Then inoculum for soil treatments was prepared by adding 5-mm diameter discs from these plates to 250 ml of potato dextrose broth. The culture was grown in a cabinet at 28 ± 2ºC with daily brief manual shaking. After 14 days, 250 ml of SDW was added to each flask, homogenized for 1 min., and then filtered through seven layers of sterilized cotton cloth. The concentration of the conidial suspension was determined and adjusted with sterile distilled water containing 0.05% (v/v) Tween 20 to give a final concentration of $2.5 x 10^6$ spores/ml, an inoculum level similar to that used by De Cal *et al*., (1997).

Evaluation of bacteria against pathogen: Five tomato seedlings cv Tiny Tim were planted in pots containing 4 litre loam–based compost (John Innes No2; Roffy Brothers, UK). Two ml of suspensions of $4 x 10^5$, $4 x 10^6$ and $4 x 10^7$ cells/ml of each bacterium were applied to each seedling in the pot to the soil in spots (10 cm diameter and depth) where seedling was planted. The disease incidence was calculated after 8 weeks by dividing the number of wilted plants by the total number of plants in all pots and multiplying by 100. Rhizosphere populations of the pathogen in the form of CFU/g of soil were determined after 4 and 8 weeks. The rhizospheric soil was only taken. The experiment was repeated three times; there was no significant difference of error variances of the three data sets on disease incidence and other parameters so data were pooled and analyzed by analysis of variance.

Effect on the plant biomass: Plants 8 weeks after planting were uprooted fresh and dry root and shoot weights were taken.

Determination of bacteria from the rhizospheric soil of the plants and pots: Soil was taken both from rhizosphere and also from the pots at 10day intervals to determine presence of *Xenorhabdus* following the method of Wollum (1982).

Results

Inhibition of fungal growth by *X. nematophila* and *Xenorhabdus* spp.: Activity was assessed qualitatively on the basis of the size of the zone of inhibition of *F. oxysporum*

f.sp., *lycopersici*. Both bacteria inhibited the growth of the pathogen with greatest inhibition at 4×10^7 cells/ml. At the lowest concentrations, the fungus showed some inhibition but had grown over the bacteria after 72 h. In the medium concentration a clear inhibition zone persisted for 72 h and was then covered by fungal hyphae. A zone of inhibition persisted for 6 days at the highest concentration (Table 1).

Effect of *X. nematophila* and *Xenorhabdus spp*. on the disease incidence and plant biomass: All treatments had significant effect on the incidence of the disease ($p\leq0.05$) and wilting decreased with increased concentration of bacterial cells (Table 2). The most effective treatment was the *Xenorhabdus* spp., at 4×10^7 cells/ml in which 26.64 % of the plants wilted compared to 39.96% wilting at the lowest cell concentration. The incidence of wilting in the plants treated with *X. nematophila* was marginally greater and ranged from 33.30 – 46.62%. There was 73.26% wilting by the *Fusarium*, only in control plants. Plants treated with the bacterial cells had significantly greater root and shoot weights than those that were not amended; the healthiest plants being those treated with the high cell concentrations but the plants treated with 4×10^7 did not show corresponding increase in plant biomass (Table 2). There was a significant difference between the species of bacteria at each concentration.

Rhizosphere populations: Rhizosphere population expressed in terms of colony forming units (CFU) of *F. oxysporum* f. sp., *lycopersici* /g soil were less in the bacterial treatments (Table 2). After 4 weeks the *Xenorhabdus* spp., and *X. nematophila* 4×10^7 cells/ml treatments showed a 97% decrease in CFU compared with the rhizosphere population of the control.

   With the increasing concentrations of bacteria there was a corresponding decrease in the CFU count of *F. oxysporum* f.sp., *lycopersici* in the rhizospheres. No bacterial cells recognisable as *Xenorhabdus* were isolated form the rhizosphere soil samples after 10 days or in succeeding samples.

Discussion

   Fusarium wilt of tomato is an intractable problem because management strategies such as cultivar resistance and chemical control are not always appropriate (De Cal *et al.,* 1995). It is not surprising that there is interest in discovering a remedy based on biological control. Recent research has shown how bacteria associated with entomopathogenic nematodes are potentially effective against a number of crop pests and diseases (Samaliev *et al.,* 2000; Vagelas *et al*., 2004).
   Several antibiotics and antimycotics from *Xenorhabdus* and *Photorhabdus* have been isolated and characterized (Paul *et al*., 1981; Nealson *et al*., 1990; McInerney *et al*., 1991a,b) and among these compounds Xenocoumacin 1, isolated from *X. nematophila* is active against animal and human pathogenic fungi (McInerney *et al*., 1991 b).
   The results of this study demonstrate the antimycotic substances produced by *Xenorhabdus* spp., inhibit growth of *F. oxysporum* f.sp., *lycopersici* and that toxins are their most likely mode of action in suppressing the pathogen. The substantial decline in the soil population of the fungus (CFU/g soil) indicates that the bacteria led to a direct inhibitory effect on the survival and multiplication of fungal propagules in the soil. However, the highest doses although effective in controlling the disease were phytotoxic so did not improve the plant biomass, the phytotoxic nature of the bacterial cells was also observed by Samaliev *et al*., (2000).

Table 1. Effect of antimycotic activity of different concentrations of bacterial suspensions of *X. nematophila* and *Xenorhabdus* spp., on the *in-vitro* survival of *F. oxysporum* fsp. *lycopersici* in Petri dishes.

Treatments	Inhibition zone
X. nematophilus	
$(4x10^5)$	+
$(4x10^6)$	++
$(4x10^7)$	+++
Xenorhabdus spp.	
$(4x10^5)$	+
$(4x10^6)$	++
$(4x10^7)$	+++
Broth only (without bacteria)	–

Note. Activity is shown on the basis of the size of the zone of inhibition of *Fol* growth on agar plates after 72 h – = no inhibition zone, + = a zone of poor fungal growth covered by bacterial inoculum, ++ = clear inhibition zone that persisted for at least for 72 then covered by fungal hyphae, +++ = clear zone of inhibition which persisted for one week

Table 2. Effect of soil application of different concentrations of bacterial suspensions of *X. nematophila* and *Xenorhabdus* spp., on the % wilt incidence, plant biomass and rhizosphere soil population of *F. oxysporum* f.sp., *lycopersici* of tomato cv. Tiny Tim under greenhouse conditions.

Treatment cells ml^{-1}	Fresh weight (g)		Dry weight (g)		Rhizosphere population (CFU/g soil)	
	shoot	Root	Shoot	Root	4 Week	8 week
46.62 (43.02)[b]	8.80c	3.40e	1.76c	2.27d	$4.5 x10^5$ b	$5.1 x 10^4$ b
39.96 (39.15)[bc]	10.76b	4.90a	2.48a	3.78a	$4.3 x 10^5$d	$4.8 x 10^3$ d
33.3 (35.15)[cd]	9.443bc	3.973c	1.717c	3.063bc	$4.5 x 10^4$ f	$3.9 x 10^2$ f
39.96 (39.15)[bc]	9.20bc	3.80cd	1.84c	2.47d	$4.4 x 10^5$c	$4.9 x 10^4$ c
33.3 (35.15)[cd]	11.20b	4.40b	2.34ab	3.40ab	$4.2 x 10^5$e	$4.4 x 10^3$ e
26.64 (30.93)[d]	10.18ab	3.463de	1.867bc	2.70cd	$4.3 x 10^4$ g	$3.7 x 10^2$ f
73.26 (58.96)[a]	3.60d	1.00f	0.74d	0.32e	$2.0 x 10^6$a	$2.0 x 10^6$ a

[*]Mean values in the same column followed by the same letter are not significantly different at p≤0.05 based on statistical analysis. The disease incidence in the parenthesis are arcsine transformed.

The failure to re-isolate *Xenorhabdus* spp., from soil 10 days after application would suggest that the bacteria were not persistent. In fact these species are only known to occur associated with entomopathogenic nematodes and how this association evolved is unknown (Forst & Clarke, 2002) and moreover Morgan *et al*., (1997) tried to isolate these bacteria from water and soil, they found that cells declined to undetectable levels after 6 days in water and after 7 days in soil.

Manipulating the rhizosphere with biological control agents will be a continuing challenge in disease management. The feasibility of applying the cells of a bacterium to a crop will need to be investigated and such decisions may depend upon the cost to benefit balance of the "product" development and registration and its overall marketability. The universal efficacy of biological control agents is less certain than that of synthetic pesticides and therefore they are less commercially attractive.

Acknowledgements

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