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Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates

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Abstract In the current study, the performance of three endophytic actinomycetes identified as *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* previously shown to reduce seedling damping-off, and root and crown rots of mature cucumber (*Cucumis sativus*) caused by *Pythium aphanidermatum* in pots under greenhouse conditions were further evaluated to determine their potential as biological control agents and as plant growth promoters in the field under the conditions of commercial production of cucumbers in the United Arab Emirates (UAE). When applied individually or in combination to cucumber seedlings, the three isolates significantly promoted plant growth and yield and reduced seedling damping-off and root and crown rots of mature cucumber plants. Individually the performance level of *S. spiralis* was relatively the best followed by *A. campanulatus* and then by *M. chalcea*. The three isolates (which were not inhibitory to each other) performed better, both as biological control agents as well as plant growth promoters, when applied together than when they were inoculated individually. The ability of these three isolates to colonize the internal tissues of roots, stems and leaves under field conditions, and to persist up to 8 weeks after seedling inoculation, showed that they can easily adapt to an endophytic habit systemically within healthy cucumber plants. As the three endophytic actinomycete isolates also colonized the rhizosphere and showed outstanding rhizosphere competency it is clear that they are facultative and not obligate endophytes. The success with the three inoculants indicated that they could well be used in place of the fungicide metalaxyl which is currently recommended for the management of *Pythium* diseases in the UAE. This is the first successful field use of endophytic actinomycetes as promising plant growth promoters and biological control agents against *Pythium* diseases of cucumber.

Keywords Biological inoculants · Disease control · Microbial interaction · Soil-borne plant pathogens

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Introduction

Species of *Pythium* are soil and water-borne and are known to be common pathogens of a variety of economically important plants (Martin and Loper 1999). *Pythium aphanidermatum* (Edson) Fitzp. causes severe seedling damping-off and root and crown rots of mature plants of cucumber (*Cucumis sativus* L.) in the United Arab Emirates (UAE). This pathogen causes major yield losses to commercial vegetable growers in this region (Stanghellini and Phillips 1975; El-Tarabily 2006; El-Tarabily et al. 2009).

The use of fungicides to manage these two diseases in the UAE has been found to be unreliable. In addition, the pathogen appears to readily develop resistance to the fungicides used. There has been in recent years, considerable interest in non-chemical control of plant diseases in the UAE, especially with current trends towards organic farming.

The use of endophytic bacteria and fungi for plant growth promotion and biological control of soil-borne plant pathogens has attracted considerable attention (e.g. Hallmann et al. 1997; Kobayashi and Palumbo 2000; Stone et al. 2000; Sturz et al. 2000; Rosenblueth and Martinez-Romero 2006; Liu et al. 2009; Wang et al. 2009). Reports on such biological control activities by endophytic actinomycetes are relatively few and include the suppression of *Fusarium oxysporum* Schlecht. Fries (Smith 1957; Cao et al. 2005), *F. pseudograminearum* Aoki and O'Donnell (Franco et al. 2007), *Verticillium dahliae* Kleb. (Krechel et al. 2002), *Rhizoctonia solani* Kühn (Krechel et al. 2002; Cao et al. 2004b; Coombs et al. 2004), *Plectosporium tabacinum* (Beyma) Palm et al. (El-Tarabily 2003), *Gauemannomyces graminis* var. *tritici* Walker (Coombs et al. 2004; Franco et al. 2007) and *Pythium* spp. (Franco et al. 2007; El-Tarabily et al. 2009).

In our recent work, we have targeted endophytic streptomycete and non streptomycete actinomycetes not only as biological control agents but also as plant growth promoters (El-Tarabily and Sivasithamparam 2006; El-Tarabily et al. 2009). In previous greenhouse screenings (El-Tarabily et al. 2009), we demonstrated the potential of three endophytic actinomycetes, namely *Actinoplanes campanulatus* (Couch) Stackebrandt and Kroppenstedt, *Micromonospora chalcea* (Foulerton) Ørskov and *Streptomyces spiralis* (Falcao de Morais) Goodfellow et al., isolated from within surface-disinfested cucumber roots, to control seedling damping-off and root and crown rots of mature cucumber plants and to promote plant growth in pots under greenhouse conditions. These taxa have to date, not been tested under commercial field production conditions in the UAE for these purposes.

These taxa had initially been selected on the basis of their ability to antagonize *P. aphanidermatum* in vitro through the screening for the production of ß-1,3, ß-1,4 and ß-1,6-glucanases, and to cause plasmolysis and hyphal lysis. Of the three isolates chosen from the greenhouse studies, only *S. spiralis* and *A. campanulatus* were capable of producing diffusible inhibitory metabolites, whilst only *S. spiralis* and *M. chalcea* produced volatile inhibitors (El-Tarabily et al. 2009). Only *A. campanulatus* parasitized the oospores of *P. aphanidermatum*. In addition, *S. spiralis* produced indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPYA), gibberellic acid (GA_3), and isopentenyl adenine (iPa). *A. campanulatus* produced IAA, IPYA, and GA_3, whilst *M. chalcea* produced only IAA and IPYA (El-Tarabily et al. 2009).

The overall objective of the present investigation was to determine whether the performance of the three outstanding endophytic actinomycetes in pots in the previous greenhouse studies (El-Tarabily et al. 2009) can be reflected in the field, under commercial production conditions, especially taking into consideration the harsh environmental conditions that exist in the Arabian Peninsula.

Field production of cucumbers in this region is undertaken on field beds under tunnel houses. This is necessary to render the field conditions conducive for vegetative and reproductive development of the cucumber crop. The previous study (El-Tarabily et al. 2009) in the greenhouse involved only evaluations of these organisms in pots on bench-tops. The specific aims were initially to compare performance of the three isolates under the two growing conditions in relation to the biological control of *P. aphanidermatum* and plant growth promotion.

In the current investigation we examined whether the in vitro antagonism towards *P. aphanidermatum* determined in the previous greenhouse studies (El-Tarabily et al. 2009) such as production of cell wall degrading enzymes (ß-1,3, ß-1,4 and ß-1,6-glucanases), hyphal lysis, oospore parasitism and antibiosis, were also related to their performance as antagonists under commercial field production conditions. Secondly, we

determined whether the ability of the three isolates to produce different plant growth regulators (PGRs) was also related to their ability to promote plant growth and increase yield under commercial field production conditions. In addition, we wanted to determine whether these isolates have the ability to colonize the internal tissues of roots, stems and leaves of inoculated seedlings and persist as endophytes up to 8 weeks after inoculation. This habit would confer the ability of the isolates to suppress the invading pathogen not only in the rhizosphere but also in the cortical tissues of the host.

Materials and methods

Two trials were carried out in a tunnel house under commercial field production conditions, one to screen against seedling damping-off and the other for root and crown rots of mature cucumber plants. The two trials were conducted in 2007 and both trials were independently repeated again in 2008.

Plant material and soil

Cucumber seeds (*Cucumis sativus* L.) cv. Cheyenne (Seminis Vegetable Seeds, Inc., Saint Louis, MO, USA) were used. The characteristics of the light-brownish loamy sandy soil used for the trials were: pH of 7.4 (in 0.01 M $CaCl_2$); electrical conductivity 0.28 dSm^{-1}; organic carbon 1.43%; the following nutrients are expressed in $mgkg^{-1}$ soil available P 492; K 238; NO_3^--N 47; NH_4^+-N 28; SO_4 37 and Fe 380.

Production of pathogen inoculum

A highly virulent isolate of *P. aphanidermatum* (CBS 116664) isolated from naturally infected cucumber roots in Al-Ain, UAE (El-Tarabily 2006) was used in the present study. Identification was confirmed by mycologists at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. *P. aphanidermatum* was maintained on potato carrot agar slants (PCA) (van der Plaats-Niterink 1981) and stored at 4°C.

To prepare the inoculum, 50 g of seeds of millet (*Panicum miliaceum* L.) was added to 30 ml of distilled water into 250 ml Erlenmeyer flasks. The flasks were autoclaved at 121°C for 30 min on three consecutive days. The millet seeds were then inoculated with eight agar plugs (6 mm diameter) from the actively growing margins of a *P. aphanidermatum* colony grown on PCA. The flasks were incubated at 28°C for 2 weeks in the dark and were shaken twice a day to ensure uniformity of colonization. Colonized millet seeds which had been autoclaved twice served as the control in non-inoculated soils.

Production of actinomycete inoculum

Actinoplanes campanulatus, M. chalcea and *S. spiralis* cultures (El-Tarabily et al. 2009) were maintained on oatmeal agar plates supplemented with 0.1% yeast extract (OMYEA) (Williams and Wellington 1982).

Aliquots (4 ml) of 20% glycerol suspension of each actinomycete were inoculated into 250 ml of starch casein broth (SCB) (Küster and Williams 1964) in 500 ml Erlenmeyer flasks and shaken on a rotary shaker (Model G76, New Brunswick Scientific-Edison, NJ, USA) at 250 rpm at 28°C for 10 days in the dark. Cells were harvested by centrifugation (12 000×*g*, at 20°C for 15 min) and the resultant pellet suspended in 10 ml sterile full phosphate-buffered saline solution (PBS) (pH 7.0) (Hallmann et al. 1997) and centrifuged again. A dilution series was made of each inoculum suspension in PBS, and 0.1 ml each of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was spread onto starch casein agar (SCA). Plates were incubated at 28°C for 10 days in the dark before determining the $cfuml^{-1}$. A concentration of approximately 10^8 $cfuml^{-1}$ of each isolate was used as inoculum.

Inoculation of seedlings with endophytic actinomycetes

Healthy cucumber seeds were surface-disinfested in 70% ethyl alcohol for 5 min followed by 1.05% solution of sodium hypochlorite (20% household bleach) for 4 min. The seeds were then washed eight times for 1 min each with sterile distilled water. Surface-disinfested seeds were germinated in autoclaved vermiculite for 3 days.

The pruned-root dip method (Musson et al. 1995) was used to inoculate the seedlings with each isolate. Briefly, when the roots were about 20 mm long, the root tips (3 mm) were trimmed using a sterilized

scalpel to facilitate the uptake of the inoculum. The seedlings were then immediately placed in sterile glass beakers at 25°C for 3 h with only their roots in contact with the inoculum suspension of each isolate (at 10^8 cfuml^{-1}). Seedlings with severed root tips exposed to autoclaved inoculum served as controls.

Treatments and lay-out for biological control and growth promotion trials

The two commercial field experiments, one for the seedlings and the other for mature cucumber plants were carried out using 11 treatments as follows: (1) control seedlings inoculated with autoclaved actinomycete inoculum in *P. aphanidermatum* non-infested soil, (2) control seedlings inoculated with autoclaved actinomycete inoculum in *P. aphanidermatum* infested soil, (3) seedlings inoculated with autoclaved actinomycete inoculum in *P. aphanidermatum* infested soil and with metalaxyl application, (4) seedlings inoculated with *A. campanulatus* in *P. aphanidermatum* infested soil, (5) seedlings inoculated with *M. chalcea* in *P. aphanidermatum* infested soil, (6) seedlings inoculated with *S. spiralis* in *P. aphanidermatum* infested soil, (7) seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in *P. aphanidermatum* infested soil, (8) seedlings inoculated with *A. campanulatus* in soil not infested with *P. aphanidermatum*, (9) seedlings inoculated with *M. chalcea* in soil not infested with *P. aphanidermatum*, (10) seedlings inoculated with *S. spiralis* in soil not infested with *P. aphanidermatum* and (11) seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in soil not infested with *P. aphanidermatum*.

For the treatment with metalaxyl (treatment 3), the soil was drenched with the fungicide (Ridomil Gold 2.5 G, Syngenta, Basel, Switzerland) at the manufacturer's recommended rate, before seedlings were transplanted. This treatment was included to compare its performance to all treatments with the actinomycetes.

The seedlings were transplanted in longitudinal rows 1 m apart within the tunnel house. Each treatment (in rows 5.5 m long×0.6 m wide) were laid out lengthwise and end to end and was separated by a buffer zone of 1.3 m long×0.6 m wide. The treatments were distributed among rows in a randomized complete block design. Each treatment was replicated four times with eight plants per treatment and a minimum of 60 cm between plants. The plants were watered using a standard commercial drip irrigation system.

Soil slabs (5.5 m length×10 cm depth×0.6 m width) were retrieved from the rows at the beginning of the experiment and mixed with *P. aphanidermatum* (0.5% w/w) (weight of colonized millet seeds/weight of soil) by mixing in a cement mixer. This inoculum density was chosen based on its suitability for a greenhouse study carried-out previously (El-Tarabily 2006). Soil with similar amounts of autoclaved infested millet seeds served as controls.

Fertilizers were incorporated in the soil slabs at the time of inoculum dispersion. Nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O) fertilizers were mixed thoroughly with soil using a cement mixer at 225, 143.1 and 259.1 kgha^{-1} in the forms of urea, single super-phosphate, and potassium sulphate, respectively. Micro-nutrients were added as chelates with EDTA at the rate of 3.5, 1.45, and 1.45 kgha^{-1}, of Fe, Mn and Zn, respectively. The treated soils were then returned to the treatment rows.

Experiment 1: Seedling damping-off

One week after soil infestation with the pathogen, cucumber seedlings inoculated with or without the actinomycete isolates using the pruned-root dip method (Musson et al. 1995) were prepared. Three-day-old seedlings were transplanted into rows in the soils containing the same 11 treatment combinations described above and the temperature within the tunnel house was maintained at 28°C±5°C. Three weeks after the seedlings were transplanted, the plants were removed from the soil, the roots washed, and the percentage of non-diseased plants (% plant survival) recorded. Disease severity was also expressed as a disease index rated on a 1–4 scale (Chen et al. 1987).

Plant growth was monitored by recording the lengths and the fresh weights of roots and shoots.

Experiment 2: Root and crown rots of mature plants

Surface-disinfested cucumber seeds were germinated in autoclaved vermiculite for 3 days. Seedlings were inoculated with each of the actinomycete suspensions using the pruned-root dip technique described above. Inoculated and non-inoculated seedlings were then

planted (5 per pot) into free-draining pots (23 cm diameter) filled with 7 kg of sieved autoclaved soil.

The pots were placed in an evaporative-cooled greenhouse at 27±2°C, watered daily to container capacity and fertilized weekly with inorganic liquid fertilizer (Thrive®) (Arthur Yates & Co Limited, Milperra, NSW, Australia) (NPK 27: 5.5: 9) at the manufacturer's recommended rate.

Once the plants were two-weeks-old, they were then transplanted into rows in the soils containing the same 11 treatment combinations described above and the temperature within the tunnel house was maintained at 28°C±5°C. Six weeks after transplanting the inoculated and non-inoculated seedlings, plants were removed, the roots washed and the plants were inspected for root and crown rot symptoms. The plant survival was recorded as percentage of non-diseased plants. Disease severity was also expressed as a disease index rated on a 1–5 scale as modified from Filonow and Lockwood (1985).

Plant growth was monitored by recording the lengths and the dry weights of roots and shoots, and number and yield of fruits (weight).

Estimation of colonization of internal tissues of roots, stems and leaves of cucumber

A trial was designed to assess the persistence of the re-introduced endophytes and to study the extent of their internal colonization of cucumber root, stem and leaf tissues 8 weeks after transplanting of the inoculated seedlings. Rifampicin-resistant mutants of the three actinomycete isolates were prepared using the method described by Misaghi and Donndelinger (1990). Rifampicin-resistant mutants were selected on OMYEA medium supplemented with 100 $\mu g ml^{-1}$ of rifampicin (Sigma Chemical Company, St Louis, MO, USA).

Healthy cucumber seeds were surface-disinfested as described above and the seeds were germinated in autoclaved vermiculite for 3 days. Seedlings were inoculated with each of the actinomycete suspensions and with a mixture of the three isolates using the pruned-root dip technique (Musson et al. 1995) described above. As controls, seedlings with severed root tips were treated with autoclaved inoculum. Inoculated and non-inoculated seedlings were then planted into field soil not infested with *P. aphanidermatum* in the tunnel house and the temperature was maintained at 28°C±5°C. Fertilizers (macro- and micro-nutrients) were incorporated in the soil before transplantation as described above in experiment 2. Each treatment was replicated four times with six plants per treatment with a minimum spacing of 60 cm between plants. The plants were watered using a standard commercial drip irrigation system.

Eight weeks after transplanting the inoculated and non-inoculated seedlings, plants were removed, the root, stem and leaf samples were washed thoroughly in tap water to remove soil particles and surface contaminants and the fresh weight recorded prior to further processing. Plant parts were surface-disinfested by first exposing them to propylene oxide vapour for 30 min (Sardi et al. 1992). They were then soaked in 70% ethyl alcohol for 5 min followed by immersion in 1.05% solution of sodium hypochlorite and shaken by hand for 4 min.

The surface-disinfested samples were then rinsed ten times (5 min each rinse) in PBS (Hallmann et al. 1997). The major key to success in isolating and studying endophytes is to ensure the plant surfaces are sterile (Hallmann et al. 1997). Therefore, sterility checks were carried out for each sample to monitor the effectiveness of the disinfestation procedures. For these checks, surface-disinfested plant pieces were dried using sterile paper towelling and pressed onto tryptic soy agar plates (TSA) (Difco Laboratories, Detroit, MI, USA) (McInroy and Kloepper 1995). In addition, 1 ml of the final buffer from the final rinse solutions were transferred to 9 ml tryptic soy broth (TSB) (Difco) and incubated at 28°C. After 4 days, an absence of bacterial growth in the sterility checks was taken to confirm sterility and actinomycetes that were isolated were considered to be endophytic.

Roots, stems and leaves were triturated in 100 ml of PBS with a sterile mortar and pestle under aseptic conditions, and then shaken for 30 min using a rotary shaker at 150 rpm. After filtering the slurry through sterile cotton cloth, the filtrate was serially diluted (10^{-2}–10^{-5}) in PBS (Hallmann et al. 1997). Aliquots (0.2 ml) were spread with a sterile glass rod over the surface of OMYEA for the enumeration of the endophytic actinomycete populations. Cooled (45°C) sterile OMYEA was amended with cycloheximide (Sigma) (50 $\mu g ml^{-1}$) and nystatin (Sigma) (50 $\mu g ml^{-1}$) immediately prior to pouring the plates to inhibit

fungal growth. Plates were dried in a laminar flow-cabinet for 15 min before incubation for 7 days in the dark at 28°C. The population dynamics of the isolates ($\log_{10}$ cfu g^{-1} fresh tissue weight) in cucumber plants (Hallmann et al. 1997) were determined using OMYEA amended with rifampicin.

Rhizosphere competence assay

The ability of the three endophytic actinomycete isolates to survive in the cucumber rhizosphere and to be rhizosphere competent was determined by the rhizosphere competence assay using the sand-non-sterilized soil tube assay described by Ahmad and Baker (1987) and modified by Nautiyal (1997) using rifampicin resistant mutants as described above. The concentration of approximately 10^8 cfu ml^{-1} of each isolate was adjusted using a spectrophotometer and was used as inoculum as described above.

For the rhizosphere competence assay, new, white polyvinyl chloride (PVC) water pipe (40 mm diameter) was cut into 25 cm lengths. Each length was cut longitudinally in half, placed together and held in place by adhesive tape. The bottom of each tube was plugged with cotton wool, filled with a sieved (3 mm) sandy non-sterile field soil and watered to container capacity. The water contained 1% water-soluble fertilizer (Thrive®) (NPK 27: 5.5: 9).

Cucumber seeds were surface disinfected as described above and the seeds were inoculated by immersion into a liquid suspension of each actinomycete isolate. Seeds were allowed to dry before sowing. Untreated seeds were used as controls.

One seed was sown in each tube to a depth of approximately 5 mm and the tubes were randomly placed (12 per box) vertically into polystyrene boxes (Polystyrene Industries, Australia) which were filled with cucumber field soil and watered to container capacity. The soil surrounding the tubes served to maintain adequate soil moisture and to reduce temperature fluctuations in the tubes over time. Approximately 1 cm of each tube protruded above the soil surface in each box. Boxes were each covered with transparent plastic bags held upright by a wire frame. No more water was added after sowing. The boxes were incubated for 3 weeks in the tunnel house maintaining the temperature at 28°C±5°C. Twelve plants per treatment were used. After 3 weeks, the tubes were opened and the roots removed. Measured from the seed, only the first 14 cm of roots were retained, these were aseptically cut into 2 cm segments and sequentially numbered from the seed (Ahmad and Baker 1987). Loose rhizosphere soil particles on the remaining root segments were carefully removed with forceps, air-dried for 24 h and added to sterile water and shaken for 1 h. The rhizosphere soil particles were numbered according to the root segments from which they were recovered. Serial dilutions were prepared (10^{-2}–10^{-3}) from which a 0.2 ml aliquot of each dilution was inoculated onto OMYEA plates. A rhizosphere soil was considered to be colonized when the colonies were detected on the plates after 7 days of incubation in the dark at 28°C.

Statistical analysis

The seedling damping-off (experiment 1) and root and crown rots of mature plants (experiment 2) were conducted in 2007 and both experiments were repeated again in 2008. Percentage data of plant survival (Tables 1 and 2) were arcsine transformed before ANOVA was carried out. All data were subjected to ANOVA and significant differences between means were determined using Fisher's Protected LSD Test at $P=0.05$. Superanova® (Abacus Concepts, Inc., Berkeley, California, USA) was used for all analyses.

Results

Biological control and growth promotion experiments

The three actinomycetes applied individually (treatments 4–6) or in combination (treatment 7) significantly ($P<0.05$) reduced damping-off of seedlings (Table 1), and the root and crown rots of mature cucumber plants (Table 2) compared to treatment 2 (pathogen alone). The most effective control was treatment 7 (all three actinomycetes applied in combination with *P. aphanidermatum*) which significantly ($P<0.05$) reduced damping-off (Table 1) and root and crown rots (Table 2) compared with the other actinomycete inoculation treatments (treatments 4, 5 and 6) where *P. aphanidermatum* was present. The performance of treatment 7 was comparable to metalaxyl application (treatment 3) in

Table 1 Experiment 1: Effects of inoculation of cucumber roots with *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* individually or in combination in soils infested with or without *Pythium aphanidermatum* on seedling damping-off and on plant growth characteristics, under commercial field production conditions in a tunnel house erected over the field beds and maintained at 28°C±5°C. Assessments were carried out 3 weeks after seedling transplantation

Treatments	[a,c]Plant survival (%)	[b,c]Disease severity	[c]Root fresh weight (g)	[c]Shoot fresh weight (g)	[c]Root length (cm)	[c]Shoot length (cm)
(1) Control seedlings in soil not infested with *P. aphanidermatum*	97.65 *e*	1.26 *abc*	2.07 *b*	5.52 *b*	8.27 *b*	7.26 *b*
(2) Control seedlings in soil infested with *P. aphanidermatum*	7.03 *a*	3.12 *g*	0.43 *a*	0.73 *a*	2.11 *a*	3.25 *a*
(3) Control seedlings in soil infested with *P. aphanidermatum* + metalaxyl	98.43 *e*	1.15 *a*	2.38 *b*	6.22 *b*	9.18 *b*	8.61 *b*
(4) Seedlings inoculated with *A. campanulatus* in soil infested with *P. aphanidermatum*	67.96 *c*	1.92 *e*	3.54 *c*	10.01 *c*	13.07 *d*	12.63 *d*
(5) Seedlings inoculated with *M. chalcea* in soil infested with *P. aphanidermatum*	57.81 *b*	2.14 *f*	3.19 *c*	8.79 *c*	11.16 *c*	10.77 *c*
(6) Seedlings inoculated with *S. spiralis* in soil infested with *P. aphanidermatum*	80.20 *d*	1.66 *d*	4.57 *d*	13.68 *d*	15.76 *e*	14.61 *e*
(7) Seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in soil infested with *P. aphanidermatum*	91.40 *e*	1.29 *bc*	5.47 *e*	16.52 *e*	17.92 *f*	16.89 *f*
(8) Seedlings inoculated with *A. campanulatus* in soil not infested with *P. aphanidermatum*	94.53 *e*	1.36 *c*	6.81 *f*	19.57 *f*	20.71 *g*	20.81 *g*
(9) Seedlings inoculated with *M. chalcea* in soil not infested with *P. aphanidermatum*	95.31 *e*	1.24 *abc*	5.76 *e*	16.75 *e*	18.61 *f*	17.92 *f*
(10) Seedlings inoculated with *S. spiralis* in soil not infested with *P. aphanidermatum*	96.87 *e*	1.20 *ab*	8.66 *g*	22.32 *g*	25.58 *h*	24.54 *h*
(11) Seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in soil not infested with *P. aphanidermatum*	97.65 *e*	1.17 *ab*	10.50 *h*	26.47 *h*	28.70 *i*	30.81 *i*

[a] Percentage data were arc-sine transformed before analysis

[b] Disease severity index was evaluated with the following scale: 1, symptomless; 2, emerged but diseased (yellowed, wilted, or visible lesions on hypocotyl); 3, post-emergence damping-off; 4, pre-emergence damping-off

[c] Values followed by the same letter within a column are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test. Values are means of eight replicate rows from two repeated independent experiments (eight plants per row for each treatment)

reducing the incidence and severity of both the diseases (Tables 1 and 2).

The three actinomycetes in the presence of the pathogen either singly (treatments 4–6), in combination (treatment 7), or in the absence of the pathogen, either singly (treatments 8–10) or in combination (treatment 11) significantly ($P<0.05$) increased the lengths and weights of roots and shoots (Tables 1 and 2), and number and yield of fruits (Table 2) compared to the controls.

Treatment 11 where all three isolates were applied together in soil not infested with the pathogen, gave the best growth promotion effect in comparison with the other treatments in both experiments (Tables 1 and 2). It is noteworthy that in treatments 4–7 in the presence of *P. aphanidermatum* and in treatments 8–11 in the absence of *P. aphanidermatum* (experiment 2), fruits were formed on the plants 2 weeks ahead of the plants in treatments 1 and 2.

Estimation of colonization of internal tissues of roots, stems and leaves

Rifampicin resistant mutants of the three isolates were re-isolated from the surface-disinfested cucumber roots, stems and leaves indicating that these strains are capable of being endophytic. The three isolates maintained their endophytic colonizing abilities and were recovered and re-isolated from

Table 2 Experiment 2: Effects of inoculation of cucumber roots with *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* individually or in combination in soils infested with or without *Pythium aphanidermatum* on root and crown rots of mature cucumber and on plant growth characteristics and fruit yield, under commercial field production conditions in a tunnel house erected over the field beds and maintained at 28°C±5°C. Assessments were carried out 6 weeks after the actinomycete-inoculated seedlings were transplanted into soil infested with or without *Pythium aphanidermatum*

Treatments	[a,c]Plant survival (%)	[b,c]Disease severity	[c]Root dry weight (g)	[c]Shoot dry weight (g)	[c]Root length (cm)	[c]Shoot length (cm)	[c]Number of fruits	[c]Fruit yield (Kg)
(1) Control seedlings in soil not infested with *P. aphanidermatum*	98.43 *e*	1.15 *ab*	1.62 *b*	9.91 *b*	16.52 *b*	71.92 *b*	19.75 *b*	2.45 *b*
(2) Control seedlings in soil infested with *P. aphanidermatum*	7.81 *a*	4.78 *g*	0.91 *a*	5.68 *a*	11.67 *a*	58.43 *a*	11.78 *a*	0.85 *a*
(3) Control seedlings in soil infested with *P. aphanidermatum* + metalaxyl	96.87 *e*	1.45 *c*	1.55 *b*	10.86 *b*	15.54 *b*	73.59 *b*	21.25 *b*	2.31 *b*
(4) Seedlings inoculated with *A. campanulatus* in soil infested with *P. aphanidermatum*	70.31 *c*	2.34 *e*	2.33 *d*	17.36 *d*	25.77 *c*	86.03 *c*	29.56 *d*	3.53 *d*
(5) Seedlings inoculated with *M. chalcea* in soil infested with *P. aphanidermatum*	62.50 *b*	2.70 *f*	2.02 *c*	13.90 *c*	23.82 *c*	83.53 *c*	26.51 *c*	3.12 *c*
(6) Seedlings inoculated with *S. spiralis* in soil infested with *P. aphanidermatum*	79.68 *d*	2.02 *d*	2.58 *e*	23.04 *e*	31.80 *d*	99.76 *d*	33.06 *e*	3.85 *e*
(7) Seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in soil infested with *P. aphanidermatum*	92.18 *e*	1.52 *c*	3.67 *g*	27.45 *f*	48.11 *g*	109.62 *e*	37.31 *f*	4.24 *f*
(8) Seedlings inoculated with *A. campanulatus* in soil not infested with *P. aphanidermatum*	99.37 *e*	1.06 *a*	3.11 *f*	28.13 *f*	39.87 *f*	127.14 *f*	44.87 *h*	4.78 *g*
(9) Seedlings inoculated with *M. chalcea* in soil not infested with *P. aphanidermatum*	98.75 *e*	1.20 *b*	2.72 *e*	24.62 *e*	36.39 *e*	115.54 *e*	40.16 *g*	4.36 *f*
(10) Seedlings inoculated with *S. spiralis* in soil not infested with *P. aphanidermatum*	97.50 *e*	1.23 *b*	3.95 *h*	32.24 *g*	46.56 *g*	138.18 *g*	48.01 *i*	5.16 *h*
(11) Seedlings inoculated with *A. campanulatus* + *M. chalcea* + *S. spiralis* in soil not infested with *P. aphanidermatum*	99.06 *e*	1.17 *ab*	4.70 *i*	39.05 *h*	51.41 *h*	157.63 *h*	51.62 *j*	5.57 *i*

[a] Percentage data were arc-sine transformed before analysis

[b] Disease severity index was evaluated with the following scale,: 1, healthy, (no discoloration of roots), plants with long taproot and many lateral roots; 2, very light infection (<25% of root area discolored, long taproot with some lateral roots; 3, slight infection (25–50% of roots discolored, moderately long taproot, no lateral roots, and slightly yellowed plants; 4, moderate infection (50–75% of roots discolored, short taproot with no lateral roots, slight stem necrosis and moderately wilted plants; 5, severe infection (>75% of roots discolored, taproot completely decayed, extensive stem necrosis with frequent girdling of stem, severely wilted and collapsed plants

[c] Values followed by the same letter within a column are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test. Values are means of eight replicate rows from two repeated independent experiments (with eight plants per row for each treatment)

healthy cucumber roots, stems and leaves after 8 weeks (Table 3). The population densities of the three isolates however were found to be less in stems and leaves compared to root samples (Table 3). The internal tissues of cucumber seedlings treated with autoclaved inoculum (control) were found to be free of any actinomycetes.

In the treatment which included the application of a mixture of the three endophytic isolates, the population density of *S. spiralis* in roots, stems and leaves was significantly ($P<0.05$) greater than that of *A. campanulatus* and *M. chalcea* (Table 3), indicating the dominance of *S. spiralis* over the two other isolates when used in combination.

Table 3 Recovery of the three endophytic actinomycetes *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* (applied individually or as a mixture) from the internal tissues of cucumber roots, stems and leaves 8 weeks after transplantation

Plant tissues	Mean population (log_{10} cfu g^{-1} fresh tissue weight)					
	A. campanulatus	*M. chalcea*	*S. spiralis*	Combined treatment		
				A.campanulatus	*M. chalcea*	*S. spiralis*
Roots	5.40 *a A*	4.79 *a B*	5.75 *a C*	4.21 *a D*	3.64 *a E*	4.87 *a B*
Stems	3.98 *b A*	3.47 *b B*	4.21 *b A*	2.95 *b C*	2.59 *b D*	3.53 *b B*
Leaves	2.43 *c BC*	2.25 *b ABC*	2.58 *c C*	2.12 *c AB*	1.94 *b* A	2.31 *c ABC*

Plants were grown for 8 weeks under commercial field conditions in a tunnel house maintained at 28°C±5°C. Values are means of 24 replicates for each treatment and the values with the same lower or same upper case letter within a column or a row, respectively are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test

Rhizosphere competence assay

Rhizosphere competence abilities of the three actinomycete isolates tested by the sand-tube method showed that rhizosphere soil particles attached to roots of cucumber seedlings were colonized to varying degrees by the actinomycete isolates (Table 4). Colonization frequency of the rhizosphere soil was greater in plants treated with *A. campanulatus* and *S. spiralis* followed by those treated with *M. chalcea* (Table 4). Population densities also showed that the isolates were detected at all sampled depths of the rhizosphere soil adhering to roots, but population densities were significantly ($P<0.05$) greater in the top 6–8 cm of the root system compared to root depths below (Table 4).

Discussion

Overall, the relative performance of the three endophytic actinomycetes in the field trials was similar to those obtained in the study conducted previously in pots under greenhouse environments (El-Tarabily et al. 2009). This shows that the ability of these isolates to be effective is not affected by the biotic and abiotic environmental variables associated with field conditions. In addition, it also indicates that the mechanisms proposed to explain their performance in pots under greenhouse conditions may also be equally operative under commercial field production conditions.

It should be noted that we used only the three best performers from the previous greenhouse studies (El-

Table 4 Population densities in log_{10} colony-forming units (cfu) for *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* in rhizosphere soil of cucumber root 3 weeks after sowing the treated seeds

Distance from seed (cm)	Population density (log_{10} cfu g^{-1} dry soil)		
	A. campanulatus	*M. chalcea*	*S. spiralis*
0–2	5.26 *a A*	4.75 *a B*	4.95 *a B*
2–4	5.20 *a A*	4.35 *b B*	4.80 *ab C*
4–6	4.75 *b A*	4.23 *b A*	4.68 *b A*
6–8	4.23 *c A*	3.76 *c B*	4.13 *c A*
8–10	4.14 *c A*	3.45 *d B*	3.87 *d C*
10–12	3.80 *d A*	2.84 *e B*	3.65 *e A*
12–14	3.55 *e A*	2.58 *f B*	3.28 *f C*

Cucumber seeds coated with each actinomycetes isolate were grown in non-sterile sandy soils, in plastic tubes placed in polystyrene boxes containing soil, in a tunnel house maintained at 28°C±5°C. Seedlings were harvested 3 weeks after sowing. Values are means of 12 replicates for each treatment and values with the same lower or same upper case letter within a column or a row, respectively are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test

Tarabily et al. 2009). The best individual performer, in relation to biological control in the current investigation, was *S. spiralis* followed by *A. campanulatus* and then by *M. chalcea*. This superiority may be related to the relatively greater ability of *S. spiralis* to endophytically colonize and persist in cucumber roots, in comparison to the other two isolates, 8 weeks after seedling inoculation (Table 3). In addition, *S. spiralis* was dominant over the two other isolates, when used in combination.

The success of *S. spiralis* over *A. campanulatus* may be also related to its ability to produce volatile metabolites as well as higher levels of ß-1,3, ß-1,4 and ß-1,6-glucanases in addition to high levels of hyphal lysis (El-Tarabily et al. 2009). Valois et al. (1996) reported that 13 soil actinomycete isolates, capable of producing ß-1,3, ß-1,4 and ß-1,6 glucanases, hydrolyzed glucans from *Phytophthora fragariae* Hickman cell walls and caused hyphal lysis. They also reported that 11 of those strains also significantly reduced the root rot disease of raspberry caused by *P. fragariae.*

Again, as with the greenhouse studies, the superior performance of *A. campanulatus* over *M. chalcea* may be attributed to its ability to produce antifungal diffusible metabolites and to parasitize *P. aphanidermatum* oospores (El-Tarabily et al. 2009). Oospores of *Pythium* spp. have been shown to promote sporulation of *Actionoplanes* spp. (Khan et al. 1993) which then aggressively parasitise them (El-Tarabily et al. 2009). *Actinoplanes* spp. from soil and rhizosphere have been successfully used to control soil-borne plant pathogens under controlled conditions against *Phytophthora megasperma* f.sp. *glycinea* Kuan and Erwin attacking soybean (e.g. Filonow and Lockwood 1985), *Pythium coloratum* Vaartaja in carrots (El-Tarabily et al. 1997) and *P. aphanidermatum* on cucumber (El-Tarabily 2006).

Mechanisms by which endophytic actinomycetes have been shown to inhibit the in vitro growth of soil-borne plant pathogens include the production of siderophores (Cao et al. 2005), cell wall degrading enzymes (e.g. chitinase (Quecine et al. 2008), ß-1,3, ß-1,4 and ß-1,6-glucanases (El-Tarabily et al. 2009)), antifungal metabolites (e.g. Smith 1957; Cao et al. 2004a, b; Naik et al. 2009; Verma et al. 2009), and the induction of systemic resistance in the host plants (Conn et al. 2006).

It should be noted that three actinomycete isolates were found to be compatible and did not appear to inhibit the activity of each other (El-Tarabily et al. 2009). The overall superiority of the mixture of the three isolates in suppressing the two diseases clearly indicates the combined effect of the three isolates tested which was as effective as metalaxyl which is currently in use in the UAE. Enhancement of the biological control activity of biological control agents have been reported in trials conducted under controlled environmental conditions, where promising isolates have been applied in combination (e.g. Mao et al. 1998; de Boer et al. 1999; Singh et al. 1999; El-Tarabily et al. 2009). Although factors identified in the previous study (El-Tarabily et al. 2009) have a significant role in the biological control activities, factors other than those assayed in that study, such as induced systemic resistance (Conn et al. 2006), may have also been involved in the performance of the tested isolates.

As with the effects related to disease suppression, the isolates were also equally efficient in enhancing plant productivity under commercial field production conditions as they were in the previous greenhouse experiments (El-Tarabily et al. 2009). In regards to plant growth promotion and yield enhancement, *S. spiralis* outperformed *A. campanulatus* and *M. chalcea* as plant growth promoters. It is noteworthy that in the in vitro studies, *S. spiralis* was capable of producing isopentenyl adenine (iPa) which was not produced by either *A. campanulatus* or *M. chalcea* (El-Tarabily et al. 2009). In addition, *A. campanulatus* which outperformed *M. chalcea* was capable of producing gibberellic acid (GA_3) (El-Tarabily et al. 2009).

Although there are many reports of production of PGRs by soil and rhizosphere microorganisms (Costacurta and Vanderleyden 1995; Arshad and Frankenberger 1998), reports of PGRs production by endophytic microorganisms are relatively few. Production in vitro of PGRs capable of promoting plant growth under controlled environmental conditions have been shown for several endophytic bacteria (e.g. Selvakumar et al. 2008; Dias et al. 2009; Sgroy et al. 2009), endophytic filamentous fungi (e.g. Khan et al. 2009) and endophytic yeasts (e.g. Nassar et al. 2005). In addition, rhizosphere and soil actinomycetes have also been shown to produce auxins (e.g. El-Tarabily et al. 2003), gibberellins (e.g. Strzelczyk and Pokojska 1984), cytokinins (e.g. El-Tarabily et al. 2003) and polyamines (e.g. Nassar et al. 2003), in vitro.

The plant growth promotion of cucumber by the three endophytic actinomycetes is noteworthy, and is similar to reports on other plant hosts where endophytic actinomycetes (e.g. Cao et al. 2005; Hamdali et al. 2008), endophytic bacteria (e.g. Ramesh et al. 2009), endophytic filamentous fungi (e.g. Mucciarelli et al. 2003, Souza et al. 2008) and endophytic yeasts (Nassar et al. 2005) were also shown to promote plant growth.

Endophytism provides unique advantages to the biological activities of endophytic actinomycetes within root tissues, where they are not only protected from the inhospitable and varying environment of the field soil but are also provided with an environment suitable for the biological activities of the metabolites they produce (El-Tarabily and Sivasithamparam 2006).

The combination of the three isolates was again superior to any of the three isolates tested individually for plant growth promotion. This trend was also found in the greenhouse studies (El-Tarabily et al. 2009) which preceded the current investigation. The previous study (El-Tarabily et al. 2009) also showed that combining effective isolates with different capabilities to produce a variety of PGRs were superior to inoculation with individual isolates.

Although the three endophytic actinomycete isolates originated from soil they showed high levels of competence not only in the rhizosphere but also as colonizers of living internal tissues of not only roots but also of stems and leaves, albeit to a lesser degree. The three isolates were successfully recovered 8 weeks after seedling inoculation. There are reports of similar endophytic colonization by actinomycetes and bacteria. Smith (1957) re-isolated *Micromonospora* sp. from tomato roots 14 days after inoculation with the same strain suggesting its endophytic residence in tomato. Pleban et al. (1995) recovered the endophyte *Bacillus cereus* Frankland & Frankland from roots and stems of cotton plants 72 days after seedling inoculation. Liu et al. (2009) reported that the endophyte *Bacillus subtilis* Cohn E1R-j was able to colonize internal tissues of wheat roots and leaves at different stages of growth after the introduction of the endophyte into the soil as soil drench.

The ability of the three actinomycete isolates to colonize cucumber rhizosphere and to be rhizosphere-competent indicates that they can challenge the invading pathogen not only in the rhizosphere but also within the root cortices where the parasitic activity of the pathogen can otherwise progress without any microbiological interference. The successful colonization of the rhizosphere in addition to the internal tissues of the roots, stems and leaves clearly indicate that these three isolates are not obligate endophytes and that they are facultative endophytes. This habit provides them with an ecological advantage for their activities as biological control agents and as plant growth promoters.

The outcome of the present study clearly indicates the potential to use these three endophytic isolates in combination under commercial field production conditions in the UAE, not only to suppress diseases caused by *P. aphanidermatum* but also to enhance crop yield. This is the first successful field use of endophytic actinomycetes as promising plant growth promoters and biological control agents against *Pythium* diseases of cucumber.

It should be noted that the employment of these isolates to suppress these diseases caused by *P. aphanidermatum* could significantly reduce the current dependency on fungicides, especially as there is a progressive interest in the adoption of organic farming strategies in the UAE.

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