INTERNATIONAL JOURNAL OF AGRICULTURE AND BIOLOGY
1560-8530/2004/06–4–599–605
http://www.ijab.org

Influence of Maize Root Colonization by the Rhizosphere Actinomycetes and Yeast Fungi on Plant Growth and on the Biological Control of Late Wilt Disease

ADEL A. EL-MEHALAWY[1], NAZIHA M. HASSANEIN, HEND M. KHATER, EL-ZAHRAA A. KARAM EL-DIN AND YOUSSEF A. YOUSSEF
Department of Microbiology, Faculty of Science, University of Ain Shams, Abbassia, Cairo–11566, Egypt
[1]Corresponding author's e-mail: adelzz1953@hotmail.com

ABSTRACT

Isolates of 85 actinomycetes and 40 yeast fungi were obtained from the rhizosphere of maize plant (*Zea mays* L.) and were screened for *in vitro* antagonism to *Cephalosporium maydis*, a causal agent of late wilt disease of maize. Of these, six actinomycetes and five yeast fungi isolates were found to be strongly antagonistic to the pathogen *in vitro*. The isolates were also examined further for their production of chitinase and β-1, 3-glucanase enzymes and antifungal metabolites as well as their ability to colonize the roots and rhizosphere of maize *in vitro* and *in vivo*. These rhizosphere isolates were competent to varying degrees in colonizing the roots of maize seedlings after 8 days on agar plates and the rhizosphere with 14 days in pots, with the competency being superior to that *Streptomyces graminofaciens, S. rochei, S. annulatus, S. gibsonii, Candida glabrata, C. maltosa,C. slooffii* and *Rhodotorula rubra*. All the six isolates of actinomycetes and five isolates of yeast fungi, significantly reduced the growth of *Ce. maydis in vitro*, but in addition the actinomycete isolates produced high levels of chitinase and β-1,3-glucanase. When the pathogen was presented as the sole carbon source, the six isolates of actinomycetes caused extensive hyphal plasmolysis and cell wall lysis. All the actinomycetes and yeast fungi isolates, individually or in combination were antagonistic to *Ce. maydis* and significantly reduced the incidence of late wilt disease when applied by seed inoculation under controlled greenhouse conditions. All the actinomycetes and yeast fungi tested in the absence of pathogen significantly increased maize plant growth measurements (fresh and dry weight of shoot and root, length of root and shoot height) as compared with the control.

Key Words: Rhizosphere; Actinomycetes; Yeast fungi; Late Wilt; Maize

INTRODUCTION

Germinating seeds and growing plant roots influence the activities of soil microorganisms in the adjoining volumes of soil known as the spermosphere and the rhizosphere, respectively (Lynch, 1990). Certain compounds in plant seed and root exudates can serve as chemoattractants leading to root colonization, such as aromatic and phenolic compounds, sugars, amino acids, organic acids and carbohydrates (Chet & Baker, 1981; Caetano-Anolles *et al.,* 1988; Nikata *et al.,* 1992; Kloepper *et al.,* 1992; van Overbeek & Elsas, 1995).

Associative root colonization by rhizosphere microorganisms that are able to grow rapidly with easily degradable substrates such as root and seed exudates, chitin, cellulose and pectins, are one of the most important factors controlling rhizosphere competence (Baker, 1991). Rhizosphere-competence assays have been developed to assess root-colonization ability, defined as colonization of root in non-sterile soil where the introduced microorganisms compete with indigenous soil microorganisms. Colonization may not be attributed to movement of these microorganisms through the rhizosphere by water, but is accounted for their proliferation (Scher *et al.,* 1984; Hanna *et al.,* 1994; Ahmad & Baker, 1997).

A current emphasis is developed on rhizosphere microorganisms (fungi & bacteria) by seed inoculation for biological control of soil-borne plant pathogens or for improvement of plant growth. Weller (1988) reported that a microorganism that colonizes root is ideal for use as biocontrol agent against soil-borne diseases, and consequently in improving plant growth (Lee *et al.,* 1990; Crawford *et al.,* 1993; Yuan & Crawford, 1995; Valois *et al.,* 1996; El-Tarabily *et al.,* 1997; Baker *et al.,* 1998; Youssef *et al.,* 2001).

The increased growth response of plants caused by rhizosphere microorganisms depends mainly on the ability of these microorganisms to survive and develop in the rhizosphere (Kleifield & Chet, 1992). Several possible plant-microbe interactions were developed which benefit plants through different mechanisms, such as the production of plant growth regulators, siderophores, phosphate solubilization, nutrient uptake and availability (Höflich & Kühn, 1996; Gupta *et al.,* 1998; Bowen & Rovira, 1999).

Accordingly, this investigation examines: (i) the efficacy of potential antagonists for the production of

chitinase, β-1, 3-glucanase, and diffusible and non-volatile antifungal metabolite(s) that cause growth minimization of *Ce. maydis in vitro*; (ii) the rhizosphere competence of each antagonist; (iii) associated disease suppression *in vivo* by seed inoculation with antagonists under controlled greenhouse conditions; and (iv) examining the efficiency on seed germination, plant biomass and growth.

MATERIALS AND METHODS

Pathogen. A virulent isolate of *Cephalosporium maydis* previously isolated from naturally infected maize (*Zea mays*, the susceptible "Baladi" variety) in Menoufia governorate (Hassanein *et al.,* 2002) was used through this study. Pure cultures were grown and maintained on potato dextrose agar and sbouraud's agar media supplemented with yeast extract.

Isolation of antagonistic rhizosphere actinomycetes and yeast fungi. Maize rhizosphere soil actinomycetes and yeast fungi were estimated using the soil dilution plate method (Johnson & Curl, 1972). They were screened for their *in vitro* antagonism to *Ce. maydis*, the causal agent of late wilt disease of maize. Of these, six actinomycetes and five yeast fungi were found to be strongly antagonistic to the pathogen. They were identified and tested for their production of antifungal compounds active against *Ce. maydis in vitro* using the dual culture agar method on Hussein's fish meal extract agar (HFMEA) (Hassanein *et al.,* 2002).

Root colonization and rhizosphere competence assays. The competence of each antagonist to colonize the rhizosphere of maize was determined in two stages. A primary indicator root colonization plate assay (Kortemaa *et al.,* 1994) was carried out *in vitro* to rapidly indicate whether or not the root exudates of maize, acting as the sole carbon sources, would support the growth of each antagonist. Successful completion was followed by a rhizosphere competence assay using a sand tube as described by Ahmad and Baker (1988).

Root colonization plate assay. Maize seeds C.V. "Baladi" were disinfected by immersion in 0.5% NaOCl for 2 min. Surface-sterilized seeds were then washed several times with sterile water and air-dried. The seeds were pre-germinated for 48 h on water agar (20 g agar L^{-1} distilled water) before being individually transferred onto water agar plates. A total of 60 seeded plates were prepared, five for each antagonist and one seed per plate. A set of five seeded plates was left as control with no further treatment. Equal amounts of each antagonist (6 actinomycetes & 5 yeast fungi) were grown on GYM-agar (4 g glucose, 4 g yeast extract, 10 g malt extract, 20 g agar & 1000 mL distilled water) plate for 1-2 weeks were inoculated each with a needle 1-2 mm from the emerging radicle (Kortemaa *et al.,* 1994). The plates were incubated in a vertical position at 25ºC. Colonization of maize roots by the antagonists was examined after 8 days. Colonization was recorded as a percentage of the total root length.

Rhizosphere competence assay. New white polyvinyl chloride (PVC) water pipe (40 mm in diameter, was cut into 25 cm lengths). Each length was cut longitudinally in half, and then the halves were 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 field capacity. The water contained 1% water-soluble fertilizer (N P K 14:6:27).

Maize seeds were surface sterilized as previously described and inoculated by immersion into a liquid suspension (1×10^{12} mL^{-1}) of each antagonist. Seeds were allowed to dry in a laminar flow cabinet for 1-2 h before sowing. Untreated seeds were used as controls. One seed was sown in each tube at a depth of approximately 5 mm and the tubes were randomly placed (12 per box) vertically into polystyrene boxes that were filled with the same disease-free maize field soil watered to field capacity. The soil surrounding the tubes served to maintain maximum 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 a transparent plastic bags held upright by a wire frame. No more water was added after sowing. The boxes were incubated for 2 weeks in a greenhouse maintained at 28ºC. Twelve plants per treatment were used. After two weeks, the tubes were opened and the roots removed. Measured from the seeds, only the first 10 cm of roots were retained; these were aseptically cut into 2 cm segments, sequentially numbered from the seed, and plated onto GYM agar plates. Loose soil particles on the root segments were carefully removed with forceps, air-dried for 24 h, then added to sterile water and shaken for 1 h. The samples of soil particles were numbered according to the root segments from which they were recovered. Serial dilutions were prepared (10^{-1}-10^{-2}), from each of which a 0.2 mL aliquot was inoculated onto GYM agar plates. All plates were incubated for 7 days in the dark at 28°C. Frequency of occurrence was assessed along the root length for all root segments and for all corresponding soil particles on which the antagonists could be detected.

Detection of antifungal activity of the antagonistic actinomycetes and yeast fungi. The antagonistic rhizosphere actinomycetes and yeast fungi were previously examined (Hassanein *et al.,* 2002) for the production of antifungal inhibitory compounds active against *Ce. maydis*. This was applied using the method of EL-Tarabily *et al.* (1996), which was also previously applied by Hassanein *et al.* (2002).

Determination of chitinase activity of the rhizosphere isolates. The isolates from rhizosphere were also examined for the production of other antifungal inhibitory compounds active against *Ce. maydis*. These isolates were screened to determine chitinase production. Each isolate was inoculated on colloidal chitin agar (CCA) and incubated at 28ºC in the dark until zones of chitin clearing were seen around

colonies. Clear zone diameters are measured (mm) and used to indicate the chitinase activity of each isolate. Large diameters (> 30 mm) represented high activity and small diameters (< 30 mm) represented low activity.

Only high active chitinase-producing (HC) isolates were screened directly against the pathogen. These isolates were streak-inoculated to one side of CCA plates and incubated for 8 days at 28ºC to promote the production and diffusion of enzymes into the medium. An agar disc (5 mm in diameter) containing actively growing *Ce. maydis* mycelia were placed on the opposite side of the inoculated plates, and they were incubated in the dark at 28ºC. *Ce. maydis* mycelial plugs were also placed on non-inoculated CCA plates as controls. Inhibition was recorded when mycelial growth of *Ce. maydis* in the direction of the HC isolate colony was retarded. The level of inhibition was calculated by substracting the distance (mm) of fungal growth radius (γ_o) of a control culture to give $\Delta\gamma=\gamma_o-\gamma$. The ratings used were modified from those of Yuan and Crawford (1995) where $\Delta\gamma$>5 mm=+; $\Delta\gamma$>10 mm=++and $\Delta\gamma$>20 mm=+++. There were four replicate plates for each isolate.

Chitinase and β-1, 3- glucanase assay. Individual 250 mL Erlenmeyer flasks containing 50 mL of minimal synthetic medium (MSM) (Tweddell *et al.,* 1994) supplemented with colloidal chitin (10 g L^{-1}) or laminarin (10 g L^{-1}) were inoculated with 1 mL of 10% glycerol suspension of each antagonist (one isolate per flask) and incubated in a gyratory shaker at 200 rpm for 5 days at 28ºC. After incubation, the suspensions from each flask were centrifuged for 30 min at 2000x*g*. The supernatant was filtered through sterile Millipore membranes of pore size 0.22 µm and collected in sterile tubes Chitinase and β-1, 3- glucanase specific activities of each antagonist were determined according to the method described by Tweddell *et al.* (1994) and modified by Singh *et al.* (1999). Chitinase activity was calculated by measuring the release of N- acetyl-D-glucosamine (NAGA) from colloidal chitin. A reaction mixture containing 1 mL of culture supernatant from a chitin-amended MSM and 1 mL of colloidal chitin (20 g L^{-1}) in 50 mm sodium acetate buffer at pH 6.8 was incubated in a water bath for 1 h at 50ºC. After boiling for 15 min, the mixture was centrifuged at 2500 g for 20 min. The concentration of NAGA in the supernatant was determined by the procedure described by Reissin *et al.* (1955). Specific activity (U=1 unit of chitinase) was defined as the amount of the enzyme that released 1 µ mol of NAGA mg^{-1} protein h^{-1}. Five replicates for each isolate were used. The specific activity of β-1, 3-glucanase was determined by measuring the amount of reducing sugars liberated from laminarin using dinitrosalicylic acid (DNA) solution (Miller, 1959). The reaction mixture contained 1 mL of culture supernatant from a laminarin-amended MSM and 0.1 mL of laminarin solution (20 g L^{-1}) in 0.2 M acetate buffer (pH 5.4). The mixture was incubated in a water bath at 40ºC for 1 h and adding 3 mL of DNS solution terminated the reaction. The colour of the end product was developed by boiling for 10 min. Reducing sugar concentration was determined by optical density at 530 nm using a scanning spectrophotometer.

Glucose was used as the calibration standard. Specific activity (U=1 unit of β-1, 3-glucanase) was defined as the amount of enzyme that released 1 µ mol of glucose mg^{-1} protein h^{-1}. Five replicates for each isolate were used. The protein content of the enzyme solution was determined by the Folin phenol reagent method (Lowry *et al.,* 1951).

Effect of antagonists on *Cephalosporium maydis* hyphae. *Cephalosporium maydis* was grown in sabouraud' s broth in 250 mL Erlenmeyer flasks containing 50 mL of broth for 10 days at 25ºC in the dark. The culture broth was removed and the mycelial mats were aseptically washed several times with sterile distilled water. A carbon-deficient salt solution described by Sneh (1981) was added to the living mycelium, which served as the sole carbon source for the antagonists. After inoculation with the chitinolytic isolates, the flasks were incubated at 25ºC in the dark and were inspected daily. Controls consisted of (i) non-inoculated carbon-deficient salt solution containing the living *Ce. maydis* mycelium only. At each sampling, a sub-sample of *Ce. maydis* hyphae was stained with lactophenol cotton blue and any subsequent changes in hyphal morphology were observed microscopically (X 40) using a light and phase contrast microscope. Six replicates for each isolate were used at each sampling.

Greenhouse trials Production of *Cephalosporium maydis* inoculum. Maize (*Zea mays*) seed-based inoculum was prepared by adding 25 g of ground seeds to 40 mL of distilled water in 250 mL conical flasks. The flasks were autoclaved at 121ºC for 30 min on three consecutive days. Small amounts of sterile distilled water were added to each flask to maintain suitable moisture before the cultivation of the pathogen. Maize ground seeds were then aseptically inoculated with 6 agars plugs (6 mm in diameter) for each flask from the actively growing margins of *Ce. maydis* and inoculated at 28ºC in the dark for two weeks. The flasks were periodically shaken to ensure uniformity of colonization. Colonized and autoclaved maize seeds served as the control. Small amounts of the colonized and control maize seeds were plated onto sabouraud's agar before use to confirm the presence or absence of *Ce. maydis*.

Preparation of antagonists inocula for the biocontrol of late wilt disease and maize growth improvement. In a previous investigation (Hassanein *et al.,* 2002) controlled maize late wilt disease by thoroughly dispersing colonized wheat bran-based inoculum in air-dry steam-pasteurized soil two weeks before adding the pathogen inoculum and seed sowing. In the present study, the antagonists' inocula were prepared by growing the antagonist on (HFMEA) to enhance the production of antifungal metabolites for 8 days at 28ºC in the dark. The growing actinomycetes and yeast fungi each was scrapped with sterile glass rod and added to 1% carboxy methyl cellulose (CMC). The sterilized seeds

were then steeped in such suspension and left overnight in sterile Petri dishes before sowing the seeds and pathogen inoculum. The treated maize seeds (C.V. Baladi) were sown (10/pot) into 25 cm diameter pots, each was filled with 3 kg of steam-pasteurized soil immediately infested with the pathogen. Seeds were sown to a depth of 3 cm in each pot and when emergence was complete (ca. 7 days) the seedlings were thinned to five per pot. Each treatment was replicated six times with five plants per replicate, in fully randomized blocks. The free draining pots were watered every other day and fertilized weekly with a liquid fertilizer (N.P.K. 14:6:27). In total there were 28 pathogen-actinomycete; pathogen-yeast fungi; pathogen alone and actinomycete and yeast fungus combinations. These were as follows: 1- 1-7: The pathogen+ each of the actinomycetes, *Streptomyces graminofaciens; S. gibsonii, S. lydicus; S. nogalater; S. rochei, S. annulatus* and a mix of the six actinomycetes. 2- 8-13: The pathogen+ each of the yeast fungi, *Candida maltosa; C. glabrata; C. slooffii; Rodotorula rubra; Trichosporon cutaneum* and a mix of the five yeast fungi. 3- 14-20: Each of the six actinomycetes and a mix of the six actinomycetes. 4- 21-26: Each of the five yeast fungi and a mix of the five yeast fungi. 5- 27: The pathogen, *Ce. maydis* alone. 6- 28: Maize plant without pathogen or antagonist.

Disease assessment. Maize plants were harvested 8 weeks after planting. The disease incidence was expressed as previously described (Hassanein *et al.*, 2002).

Statistical analysis. A randomized complete block design was used and analysis of varience was carried out using Superanova® (Abascus Concepts, lnc. Barakeley, California, USA) to evaluate the effect of antagonists on late wilt disease development in greenhouse trials. Percentage data (disease incidence & root colonization) were arcsine transformed before analysis of varience was carried out. Significant differences between means were determined by Fisher's Protected LSD Test at P= 0.05. Analysis of variance was also carried out to evaluate the effect of each antagonist on the growth promotion of maize plant. Results are means of 30 replicate plants for each treatment.

RESULTS

Root colonization plate assay. In the plate tests root colonization ability of six actinomycetes and five yeast fungi rhizosphere isolates was clearly observed but with different degrees. In general, root colonization by the actinomycete isolates was greater than that of the yeast fungi isolates. Root colonization frequency for *S. graminofaciens, S. rochei, S. annulatus* and *S. gibsonii* was greater than that of *S. lydicus* and *S. nogalater*. They had colonized 92 to 95% of roots by 4 days after radicle emergence. Similarly, root colonization frequency for *C. glabrata, C. maltosa, C. slooffii* and *Rhodotorula rubra* was also greater than that for *Trichosporon cutaneum*. They had colonized 85 to 90% of roots after 7 days.

Rhizosphere competence assay. Root and attached soil particles of 14 day-old maize seedlings were colonized to different degrees by the actinomycetes and yeast fungi isolates with the frequency of colonization being significantly ($P < 0.05$) greater in the first 2 cm of root and soil nearest to the seed. Root colonization frequency in the root segments and in the rhizosphere soil was greater in *S. graminofaciens, S. rochei, S. annulatus, S. gibsonii, C. glabrata, C. maltosa* and *C. slooffii* treated plants, followed by rest of the actinomycetes and yeast fungi treated plants, respectively (Table I).

Effects of antagonists on *Cephalosporium maydis* hyphae. When the living mycelium of *Ce. maydis* served as a sole carbon-deficient salt solution, the six actinomycetes induced plasmolysis after 15 h incubation and lysis of the hyphae of the pathogen becoming evident after 3 days of incubation. Mycelial mats in non-inoculated carbon-deficient salt solution or in flasks inoculated with non-chitinolytic yeast fungi isolates remained healthy and intact.

Chitinase and β-1, 3-glucanase assays. The six antagonistic actinomycetes, grew on MSA containing colloidal chitin and laminarin. The chitinase specific activities were 5.50 (*S. graminofaciens*), 5.30 (*S.rochei*), 5.10 (*S. annulatus*), 5.00 (*S. gibsonii*), 4.80 (*S. lydicus*), 4.20 (*S. nogalater*), whilst the β-1, 3-glucanase-specific activities for the same species of actinomycetes were 0.76, 0.71, 0.70, 0.68, 0.59 and 0.46 U, respectively.

Table I. The effect of selected rhizosphere actinomycetes and yeast fungi on the incidence of *Cephalosporium maydis* late wilt disease of maize under greenhouse conditions

Treatments	Disease Incidence (%)	Percentage Control (%)
1- Un-infested control	0^a	100^a
2- Pathogen alone	94^b	6^b
3- Pathogen + *Streptomyces graminofaciens*	4^c	96^c
4- Pathogen + *S.gibsonii*	6^c	94^c
5- Pathogen + *S. lydicus*	6^c	94^c
6- Pathogen + *S. nogalater*	7^c	93^c
7- Pathogen + *S. rochei*	4^c	96^c
8- Pathogen + *S. annulatus*	5^c	95^c
9- Pathogen + Mix. of six actinomycetes	2^c	98^c
1O- Pathogen + *Candida glabrata*	5^c	95^c
11- Pathogen + *C. maltosa*	5^c	95^c
12-Pathogen + *C. slooffii*	5^c	93^c
13- Pathogen + *Rhodotorula rubra*	10^d	90^d
14- Pathogen + *Trichosporon cutaneum*	11^d	89^d
15- Pathogen + Mix. of five yeast fungi	3^c	97^c

Percentage data of disease incidence were arcsine transformed before analysis. Values with The same letter within a column are not significantly ($P > 0.05$) different according to Fischer's Protected LSD Test. Results are means of 30 replicates for each treatment

Table II. The effect of six actinomycetes and their mixture on the growth of maize

Actinomycetes	Length of root (cm)	Height of shoot (cm)	Fresh weight of shoot (g)	Fresh weight of root (g)	Dry weight of shoot (g)	Dry weight of root (g)
Streptomyces graminofaciens	28.07±(0.49)^{c}	52.64±(0.89)^{d}	22.85±(0.52)^{de}	13.49±(0.12)^{b}	4.27±(0.13)^{e}	2.28±(0.08)^{b}
S. gibsonii	26.07±(103)^{b}	45.28±(0.92)^{b}	19.37±(0.36)^{c}	13.93±(0.36)^{b}	3.27±(0.13)^{b}	2.29±(0.07)^{b}
S. lydicus	24.57±(0.68)^{b}	5O.14±(0.63)^{c}	22.55±(0.58)^{d}	12.82±(0.04)^{b}	3.31±(0.12) ^{b}	2.27±(0.08)^{b}
S. nogalater	25.92±(0.69)^{b}	53.64±(1.05)^{d}	24.05±(0.06)^{ef}	12.87±(0.07)^{b}	4.25±(0.12)^{c}	2.30±(0.09)^{b}
S.rochei;	25.71±(0.73)^{b}	53.50±(1.04)^{d}	17.48±(0.45)^{b}	12.78±(0.08)^{b}	3.28±(0.12)^{b}	2.24±(0.08)^{b}
S. annulatus	29.21±(0.48)^{c}	59.71±(0.79)^{e}	24.27±(0.37)^{f}	13.87±(0.12)^{b}	4.69±(0.10)^{d}	2.4O±(0.08)^{b}
Mix. of actinomycetes.	30.14±(0.61)^{d}	51.O7±(0.45)^{f}	21.71±(0.53)^{d}	13.59±(0.10)^{b}	4.63±(0.10)^{c}	2.34±(0.08)^{b}
Cont.	15.71±(0.61)^{a}	30.07±(0.78)^{a}	11.90±(0.69)^{a}	3.63±(0.O8)^{a}	2.36±(0.O8)^{a}	0.83±(0.09)^{a}

Values with the same letter within a column are not significantly different (P > 0.05) according to Fischer's Protected LSD Test. Results are means of 15 replicates for each treatment. The values in parentheses are the standard error of the mean

Table III. The effect of the five yeast fungi and their mixture on the growth of maize

Yeast fungi	Length of root (cm)	Height of shoot (cm)	Fresh weight of shoot (g)	Fresh weight of root (g)	Dry weight of shoot (g)	Dry weight of root (g)
Candida glabrata	31.35±(0.56)^{e}	37.92±(1.02)^{bc}	23.74±(0.79)^{e}	13.30±(0.11)^{b}	3.29±(0.13)^{b}	2.25±(0.09)^{b}
C. maltosa	26.78±(0.61)^{b}	38.93±(0.89)^{d}	21.25±(0.68)^{b}	13.38±(0.13)^{b}	3.25±(0.13)^{b}	2.26±(0.08)^{b}
C. slooffii	26.00±(0.4)$^{b\backslash}$	36.71±(0.46)^{b}	19.31±(0.44)^{c}	12.86±(0.06)^{b}	3.26±(0.12)^{b}	2.32±(0.08)^{b}
Rhodotorula rubra	2.8.5±(0.32)^{c}	4600±(0.56)^{de}	17.78±(0.44)^{bc}	12.8±(0.05)^{b}	3.29±(0.12)^{b}	2.30±(0.01)^{b}
Trichosporon cutaneum	29.71±(0.53)^{d}	47.60±(0.67)^{e}	16.87±(0.42)^{b}	12.7±(0.08)^{b}	3.23±(0.11)^{b}	2.23±(0.09)^{b}
Mix. of the five yeast.	26.56±(0.41)^{b}	45.21±(1.10)^{d}	17.72±(0.46)^{bc}	12.47±(0.009)^{b}	3.30±(0.11)^{b}	2.25±(0.08)^{b}
Control	17.92±(0.54)^{a}	30.92±(0.69)^{a}	11.21±(0.33)^{a}	3.65±(0.08)^{a}	2.40±(0.08)^{a}	0.83±(0.09)^{a}

Values with the same letter within a column are not significantly different (P > 0.015), according to Fischer's Protected LSD Test. Results are means of 15 replicates for each treatment. The values in parentheses are the standard error of the mean

Greenhouse trials Biocontrol of late wilt disease by seed inoculation. Plants in soil not infected by the pathogen did not show any disease symptoms (Tables II & III). Treatment that contained *Ce. maydis* and the antagonistic species of actinomycetes or yeast fungi, individually or in combination, significantly ($P < 0.05$) reduced the incidence of the wilt disease of maize caused by *Ce. maydis* compared to the treatment which includes the pathogen alone. There were significant differences in the disease index, the number of diseased maize seedlings between maize grown in each of the actinomycete and yeast fungus + pathogen infested soil treatments and the pathogen in infested soil only (Table I). *Ce. maydis* was recovered on Sabouraud's medium amended with Streptomycin from diseased maize plants in all infested pots. The application of each of the antagonistic actinomycetes or the yeast fungi or each of their mixture by seed inoculation increased the percentage of control of late wilt disease, in a range exceeded that obtained when these antagonists were applied by soil infestation as in a previous work (Hassanein *et al.,* 2002). The actinomycetes and yeast fungi, *S. graminofaciens, S. rochei, S. annulatus, S. gibsonii*, *C. glabrata, C. maltosa* and *C. slooffii,* were the most aggressive antagonists followed by the other antagonistic species. The application of mixture of the six actinomycetes or the mixture of the five yeast fungi increased the percentage of control of late wilt disease in a range exceeded that obtained when these antagonists were applied singly.

In the absence of pathogen, the antagonists alone or in combination had no harmful effects on seed germination or plant growth. The antagonistic rhizosphere actinomycetes and yeast fungi were assessed for their effects upon maize seed germination, growth and development (Table II & III). Maize plant growth measurements were used to assess the potential impact of the different inocula of the actinomycetes and yeast fungi on maize growth. In the absence of the pathogen, each of the six actinomycetes and the five yeast fungi and their mixtures significantly improved maize growth by increasing all maize growth measurements. These rhizosphere antagonists significantly increased maize fresh and dry weight of shoot and root, length of root; shoot height as compared to the control (Tables II & III).

DISCUSSION

This study is the first record of control of late wilt disease of maize caused by *Cephalosporium maydis* by rhizosphere actinomycetes and yeast fungi Data revealed that the six actinomycetes and yeast fungi isolates which were found to be strongly antagonistic to *Ce. maydis in vitro* (Hassanein *et al.,* 2002) were effective producers of antifungal metabolites. In addition, it was found that the isolates of actinomycetes produced chitinase and β-1, 3-glucanase and caused extensive plasmolysis and cell wall lysis of *Ce. maydis in vitro*.

Since the cell wall of *Ce. maydis* consists largely of chitin and β-glucanse (Bartnicki-Garcia & Lippman, 1982), there is a voluminous body of literature on the ability of actinomycetes and fungi to parasitize spores, hyphae and other fungal structures, and many of these observations are linked with plant disease biocontrol (Jeffries & Young,

1994; van den Booger & Deacan, 1994; Davanlou *et al.,* 1999). For example, indirect population dynamic studies showed that mycelium of *Rhizoctonia solani* in the rhizosphere of potato was a prerequisite for development of the mycoparasite *Verticillium biguttatum* (van den Boogert & Velviss, 1992), and rhizosphere competence was strongly related to biocontrol in mycoparasite isolates of *Trichoderma* species (Sivan & Harman, 1991; Thrane *et al.,* 1997; Harman & Bjorkman, 1998). As the maize root is the most obvious region for symptom development of the wilt disease in maize, and the pathogen *Ce. maydis* is seed borne (Hassanein *et al.,* 2002), the roots may also play a critical part in the infection process. Gragan *et al.* (1980), Pettersan and Gragan (1985) and Subbarao (1998) indicated the importance of a competence depth of up to 8 cm in relation to infection of lettuce by *Sclerotinia minor*. In the present study the non-chitinolytic rhizosphere yeast fungi were also capable of producing detectable levels of diffusible antifungal metabolites *in vitro*. We surmised from these results that the main mechanism involved in late wilt disease reduction by the six actinomycetes and five yeast fungi is the production of non-volatile diffusible metabolites, since the production of these metabolites was related to significant *in vitro* inhibition and biological control of the pathogen. In addition, the six actinomycetes, which were capable of producing antifungal metabolites, can also produce chitinase and β-1, 3-glucanase, yet were rated in their ability to control the pathogen in greenhouse experiments. Once cell wall damage has occurred, the pathogen is more likely to be susceptible to attack by other biological, physical and chemical agents. The antagonistic isolates of actinomycetes and yeast fungi in the present study were capable of growing totally at the expense of the hyphae of *Ce. maydis*, indicating their potential for pathogen suppersion (Simon & Sivasithamparam, 1988; El-Tarabily *et al.,* 2000; Hassanein *et al.,* 2002) where the antagonism takes place outside the limits of rhizosphere.

This study is the first record of late wilts disease of maize caused by *Ce. maydis* being controlled by the antagonistic rhizosphere streptomycete actinomycetes and yeast fungi (Table I). The mechanism involved in disease reduction appears mainly to be antibiosis for both the actinomycetes and yeast fungi. The actinomycete isolates were capable of parasitizing *Ce. maydis* hyphae in addition to producing antifungal metabolites. Most studies have utilized actinomycetes and yeast fungi as potential biological control agents of plant pathogen (Yuan & Crawford, 1995; Takaki *et al.,* 1996; Benbow & Sufar, 1999; Buck & Adrews, 1999). Many actinomycetes species used in the biocontrol of plant pathogens were by producing antibiotics (Locci & Schofield, 1989; You *et al.,* 1996). On the other hand, yeast fungi applied in the control of plant pathogens were found to produce toxins (killer toxins) (Hodgson *et al.,* 1995; Abranches *et al.,* 1997).

In the present investigation, the selected isolates of actinomycetes and yeast fungi significantly stimulated maize root and stem growth measurements compared with untreated control and with the treatment, which included the pathogen alone. This was observed for all treatments with each of the six actinomycetes and each of the five yeast fungi and their combination (Table II & III). This is the first record of these rhizosphere microorganisms producing promoting plant growth. This again could be an indication that the actinomycetes and yeast fungi were producing growth factors. These metabolites are considered important after being taken up by the plant or indirectly by modifying the rhizosphere environment. Höflish and Kühn (1996) reported that the promotion of cruciferous oil and intercrops and nutrient uptake was stimulated by inoculating rhizosphere microorganisms. Also rhizosphere actinomycetes and yeast fungi promoted plant growth by oxidizing ammonium to nitrate, oxidizing elemental sulphur to sulphate and solubilizing insoluble phosphate.

This study has further revealed that certain actinomycetes and yeast fungi can reduce the late wilt disease of maize caused by *Ce. maydis* under the conditions employed in the current investigation. This work was carried out under controlled glasshouse conditions with steam-pasteurized soil and although this is common practice, steam-pasteurization could drastically affect these results. Pasteurized soil was used with the aim of disinfesting the soil of resident fungal pathogen. Field trials are now in progress to further evaluate this potential.

REFERENCES

Ahmad, J.S. and R. Baker, 1988. Rhizosphere competence of *Trichoderma harzianum. Phytopathol.,* 77: 182–9

Abranches, J., P.B. Morais, C.A. Rosa, H. Mendonca, L.C. Hagler and A.N. Hgler, 1997. The incidence of killer activity and extracellular proteases in tropical yeast communities. *Candian J. Microbiol.,* 43: 328–36

Baker, R., 1991. Diversity in biological control. *Crop Protection,* 10: 85–94

Bucki, P.M., F.S. Laich, A.L. Melegari and A.R. Escande, 1998. Dampimg off of egg plant (*Solanum melongena* L.): isolation of causal agents and selection of microorganisms for its biological control. *Fitopathol.,* 33: 115–23

Bown, G.D. and A.D. Rovira, 1999. The rhizospher and its management to improve plant growth. *Adv. Agron.,* 66: 1–102

Bartnicki-Garcia, S. and E. Lipman, 1982. Fungal cell wall composition. *In*: Laskin, A.L., H.A. Lechevalier (eds.), pp: 229–52. *CRC Handbook of Microbiolgy*, vol. 4, Boca Raton, Fl: CRC Press, USA

Benbow, J.M. and D. Sugar, 1999. Fruit surface colonization and biological control of post-harvest diseases of pear by pre-harvest yeast application. *Plant Disease,* 3: 839–44

Buck, J.W. and J.H. Andrews, 1999. Role of adhesion in the colonization of leaves by yeast *Rhodosporidium toruloides*. *Candian J. Microbiol.,* 45: 433–40

Chet, L. and R. Baker, 1981. Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. *Phytopathol.,* 71: 286–90

Crawford, D.L., J.M. Lynch, J.M. Whipps and M.A. Ousley, 1993. Isolation and characterization of actinomycetes antagonists of a fungal root pathogen. *Appl. Environ. Microbiol.,* 59: 3899–3905

Caetano-Anolles, G., D.K. Christ and W.D. Baver, 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin reqires functional nodulation genes. *L. Bacteriol.,* 170: 3164–69

Davanlou, M., A.M. Madsen, C.H. Madsen and J. Hockenhull, 1999. Parasitism of macroconidia, chlamydospores and hyphae of *Fusarium culmorum* by the mycoparasite *Pythium* species. *Plant Pathol.,* 48: 352–9

El-Tarabily, K.A., Hardy GEStJ, K. Sivasithamparam, A.M. Hussein and D.I. Kurtböke, 1997. The potential for the biological control of cavity-spot disease of carrot, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. *New Phytopathologist,* 137: 459–507

El-Tarabily, K.A., M.L. Sykes, L.D. Kurtböke, H. Gestj, A.M. Barbosa and R.F.H. Dekker, 1996. Synergistic effects of a cellulose-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot Banksia grandis. *Candian J. Bot.,* 74: 618–24

El-Tarabily, K.A., M.H. Soliman, A.H. Nassar, H.A. Al-Hussani, K. Sivasithamparam, F. McKenna and Hardy GEStJ, 2000. Biologicalcontrol of *Scelrotini minor* using a chitinolytic bacterium and actinomycetes. *Plant Pathol.,* 49: 73–583

Gupta, A., M. Gopal, A. Pandey, C.R. Soccol, V.K. Josk, A.K. Saxena and K.V.B.R. Tilak, 1998. Bacterisation of green gram with rhizosphere bacteria for enhanced plant growth. *J. Sci. Indust. Res.,* 57: 126–31

Grogan, R.G., M.A. Sall and Z.K. Punja, 1980. Concepts for modeling root infection by soil-borne fungi. *Phytopathol.,* 70: 361–63

Hanna, K., R. Hannu, H. Kielo, and S. Alno, 1994. Root colonization ability of antagonistic *Streptomyces griseoviridis*. *Plant Soil,* 163:77–83

Hassanein, N.M., A.A. El-Ehalawy, H.M. Khater, A.K. El-Zahraa and A.Y. Youssef, 2002. The potential of selected rhizosphere actinomycetes and yeast fungi for the biological control of late wilt disease of maize caused by *Cephalosporium maydis. African J. Mycol. Biotech*., 1: 167–88

Hoflich, G. and G. Kühn, 1996. Promotion of plant growth and nutrient uptake of cruciferous oil and intercrops by inoculated rhizosphere microorganisms. *Zeitscrift fur Pflanzenernährung und Bodenkunde,* 159: 575–81

Harman, G.E. and T. Björkman, 1998. Potential and existing uses of Trichoderma and Gliocladium for plant disease control and plant growth enhancement. *In*: Harman G.E., C.P Kubicek (eds.) *Trichoderma* and *Gliocladium*, Vol. 2. *Enzymes, Biological Control and Commercial Applications*, pp: 229–65. Taylor Francis, London

Hodgson, V.J., D. Button and G.M. Walker, 1995. Anti-Candida activity of a novel killer toxin from the yeast *Williopsis mrakii*. *Microbiol.,* 141: 2003–12

Johnson, L.F. and E.A. Curl, 1972. *Methods for Research on the Ecology of Soil-Borne Plant Pathogens.* Burgess Publication Co., Minneapolis, USA

Jeffries, P. and T.W.K. Young, 1994. *Inter-Fungal Parasitic Relationships.* Wallingford, CAB International, UK

Kloepper, J., S. Tuzun and J. Kuc, 1992. Proposed definitions related to induced disease resistance. *Bio. Sci. Tech.,* 2: 347–9

Kleifield, O. and I. Chet, 1992. *Trichoderma harzianum*-interaction with plants and effect on growth response. *Plant Soil,* 144: 267–72

Kortemaa, H., H. Rito, K. Heahtela and A. Smolandder, 1994. Root colonization ability of antagonistic *Streptomyces griseoviriling*. *Plant Soil,* 163: 77–83

Krankenberger, W.T., A.C. Chang and M. Arshad, 1990. Response of *Raphanus sativus* to the auxin precursor, L-triptophan applied to soil. *Plant Soil,* 129: 235–41

Lynch, T.M., 1982. The rhizosphere. *In:* Burns, R.G. and J.M. Slaters (eds.), *Experimental Microbial Ecology*. Blackwell Scientific Publications, London

Lee, I.K., C.J. Kim, S.D. Kim and I.D. Yoo, 1990. Antifungal antibiotic against fruit rot disease and red pepper from *Streptomyces parvullus*. *Korean J. Appl. Microbiol. Biotech.,* 18: 142–7

Locci, R. and G.M. Schofield, 1989. Genus *Streptoverticillium bladacci*. *In:* Williams, S.T., M.E. Sharpe and J.G. Holt (eds.). *Bergey's Manual of Systematic Bacteriology* (Vol. 4) pp: 2492–504. Williams and Wilkins, Baltimore, USA

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* 193: 265–75

Miller, G.L., 1959. Use of dinitro-salicylic acid reagent for determination of reducing sugar. *Anal. Chem.,* 31: 426–8

Nikata, T., K. Sumida, J. Kats and H. Ohtake, 1992. Rapid method for analysing bacterial behavioural responses to chemical stimuli. *Appl. Environ. Microbiol.,* 58: 2250–4

Petterson, C.I. and R.G. Grogan, 1985. Differences in epidemiology and control of lettuce drop disease caused by *Sclerotinia minor* and *S. sclerotiorum*. *Plant Disease,* 69: 766–70

Reissing, J.L., J.L. Strominger and L.F. Leloir, 1955. A modified colorimetric method for the estimation of N-acetylamino sugar. *J. Biol. Chem.,* 27: 959–66

Scher, F.M., J.S. Ziegle and J.W. Kloepper, 1984. A method for assessing the root-colonizating capacity of bacteria on maize. *Candian J. Microbiol.,* 30: 151–7

Singh, P.P., Y.C. Shin, C.S. Park and Y.R. Chung, 1999. Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. *Phytopathol.,* 89: 92–9

Sneh, B., 1981. Use of rhizosphere chitinolytic bacteria for biological control of *Fusarium oxysporum* f.sp. *dianthi* in carnation. *J. Phytopathol.,* 100: 251–6

Sivan, A. and G.E. Harman, 1991. Improved rhizosphere competence in protoplast fusion progeny of *Trichoderma harzianum*. *J. Gen. Microbiol.,* 137: 23–9

Subbarao, K.V., 1998. Progress toward intergrated management of lettuce drop. *Plant Disease,* 82: 1068–78

Simon, A. and K. Sivasithamparam, 1988. Interactions among Gaeumannomyces graminis var. tritici, Trichoderma koningii and soil bacteria. *Canadian J. Microbiol.,* 34: 871–6

Tweddell, R.J., S.H. Jabaji-Hare and P.M. Charest, 1994. Production of Chitinase and β-1,3- glucanases by *Stachbotrys elegans*, a mycoparasite of *Rhizoctonia solani*. *Appl. Environ. Microbiol.,* 60: 489–95

Takaki, S., A. Kitamura, T. Marumota, D. Ishida and S. Tanaka, 1996. Control of *Fusarium* disease using antagonistic actinomycetes. III. Effect of a microbial inoculum (Material A) on microbial flora under some soil environmental conditions and development of effective method of application to radish yellows. *Soil Microorganisms,* 47: 51–8

Thrane, C., A. Tronsmo and D. Funck-Jensen, 1997. Endol-1, 3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction and biological activity against plant pathogenic *Pythium* spp. *European J. Plant Pathol.,* 103: 331–44

Valois, D., K. Fayed, T. Barasubye, M. Garon, C. Dery, R. Brezezinski and C. Eaulieu, 1996. Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root. *Appl. Environ. Microbiol.,* 62: 1630–5

Van Den Boogert, P.H.J.F. and H. Velvis, 1992. Population dynamics of the mycoparasite *Verticillium biguttatum* and its host, *Rhizoctonia solani*. *Soil Biol. Biochem.,* 24: 157–64

Van Den Boogert, PHJF and J.W. Deacon, 1994. Biotrophic mycoparasitism by *Vericillium biguttatum* on *Rhizoctonia solani*. *European J. Plant Pathol.,* 100: 137–56

Van Overbeek, L.S. and J.D. Van Elsas, 1995. Root exudates-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. *Appl. Environ. Microbiol.,* 61: 890–8

Weller, D.M., 1988. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Ann. Rev. Phytopathol.,* 26: 379–407

Yuan, W.M. and D.L. Crawford, 1995. Characterization of *Stretomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. *Appl. Environ. Microbiol.,* 61: 3119–28

Youssef, Y.A., K.A. El-Tarabily and A.M. Hussein, 2001. *Plectosporium tabacinum* root rot disease of white lupine (*Lupinus termis* Forsk.) and its biological control by *Streptomyces* species. *J. Phytopathol.,* 1490: 29–33

You, M.P., K. Sivasithamparam and D.L. Kurtböke, 1996. Actinomycetes in organic mulch used in avocado plantations and their ability to suppress *Phytophthora cinnamomi*. *Biol. Fertil. Soil.,* 22: 237–42

(Received 25 February 2004; Accepted 22 March 2004)