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Full Length Research Paper

Colonization of exopolysaccharide-producing *Paenibacillus polymyxa* on peanut roots for enhancing resistance against crown rot disease

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The effect of *Paenibacillus polymyxa* (syn. *Bacillus polymyxa*) which produces an exopolysaccharide (EPS) on control of crown rot disease caused by *Apergillus niger* of peanut was investigated. In an *in vitro* assay, two strains of *P. polymyxa* (B5 and B6) were tested against *A. niger*. Both strains showed inhibitory effect against *A. niger.* Growth, protein and biopolymers production of bacteria were evaluated. The biopolymers were purified with several precipitation steps using ethanol and cetyl-trimethyl-ammonium bromide. Carbohydrate analysis using various color reactions, infrared spectroscopy, and high performance liquid chromatography (HPLC) revealed that the biopolymer is a homopolysaccharide, which is consisting of various sugars such as glucose, galactose, mannose and xylose. When these strains of *P. polymyxa* were applied to seed and sowed in soil infested with *A. niger*, they significantly suppressed crown rot disease development and decreased survival of the *A. niger* pathogen. Over a period of 60 days, the population of bacteria was greatly increased. The bacterium colonized plant roots and were able to migrate downward with the root as it elongated. Scanning electron microscopic analysis of one month plants from seeds previously inoculated with *P. polymyxa* revealed dense colonization on the roots. Moreover, significant increases in activities of plant defense enzymes include β-1,3-glucanase and chitinase were recorded in treated roots compared with untreated. *In vivo*, two trials were conducted in 2005 and 2006 seasons to define the effect of bacterium treatment on crown rot disease control and pod yield. Plants grown from peanut seeds previously treated with *P. polymyxa* displayed significant resistance to the crown rot disease triggered by *A. niger*. Coating seeds with bacterium decreased infection by *A. niger*, *Aspergillus* counts in the rhizosphere, pods and seeds as well as increased root colonization with bacteria. The yield of bacterium-treated peanut plants was significantly higher than untreated control plants. These results showed that *P. polymyxa* is potentially a biocontrol agent for use in controlling of *A. niger* in roots and seeds of peanut plants.

Key words: *Apergillus niger*, exopolysaccharide, crown rot, *Paenibacillus polymyxa*, peanut.

INTRODUCTION

Crown rot disease of peanut (*Arachis hypogaea* L.) caused by the highly virulent strain of *Asperigullus niger* Teigh is a very important plant disease in Egypt (Wafaa and Abo Sedra, 2000; Elwakil 2003) and several other tempe-rate countries (Phipps, 2000; Carina et al., 2006). Peanut seeds infected by seed-borne fungi have been reported to produce seed abortion, shrunken seeds, reduce seed size, seed rot, seed necrosis, seed discolor-ration, reduction of germination capacity and physiologi-cal alternation of seed (Elwakil, 2003). The disease is seed-borne and survives on infected peanut seeds (Carina et al., 2006). Due to environmental concerns, there is considerable interest in finding alternatives to chemical pesticides for suppression of soilborne plant pathogens. Biological control represents an attractive alternative for the future because of the many concerns about the pesticides use. Ideally, an agent of biological control of fungal root pathogens should exert a sufficient

amount of antagonistic activity in the rhizosphere to significantly reduce root disease symptoms.

Paenibacillus polymyxa (=*Bacillus polymyxa*; Ash et al., 1994), a common soil bacterium belongs to the group of plant growth-promoting rhizobacteria (PGPR) (Timmusk et al., 1999; Selim et al., 2005). A range of activities has been found to be associated with *P. polymyxa*-treatment, some of which might be involved in plant growth promotion (Timmusk et al., 2003). Indirect promotion of plant growth occurs when PGPR antagonize or prevent the effects of phytopathogens or deleterious microorganisms. Most mechanisms proposed to explain indirect growth promotion suggest that the active principle may be a secondary bacterial metabolite which antagonizes pathogens. *P. polymyxa* is known to produce antibiotic compounds, and inoculation with *P. polymyxa* suppressing several plant pathogens (Bloemberg and Lugtenberg, 2001; Beck, 2003, Selim et al., 2005, Svetoch et al., 2005; Lorentz et al., 2006). It has been isolated from the rhizospheres of white clover, perennial ryegrass, crested wheatgrass (Holl et al., 1988) and green bean (Petersen et al., 1996) cucumber (Roberts et al., 2005) and garlic (Kajimura and Kaneda 1996). Induced resistance can also be as a result of root colonization by PGPR (Timmusk et al., 2003). The latter response is called induced systemic resistance (ISR), and has been shown to protect against disease in several plant species (Timmusk et al., 1999; Timmusk et al., 2003). Effective colonization of plant roots by PGPR plays an important role in growth promotion, irrespective of the mechanism of action (Davey and O'toole, 2000). Bacterial exopolysaccharides (EPS) can protect bacteria from various stresses (Amellal et al., 1998). Lipopolysaccharides from bacterial outer membranes have been proposed to be involved in the induction of ISR (Van Peer and Schippers, 1992). In recent years, much attention has been paid to microbial polysaccharides due to their unique properties and the possibility of easy establish and quick mass production. Furthermore, several microbial polysaccharides have been reported to possess specific physical properties and physiological activities (Amellal et al., 1998).

In this study, we have isolated. *P. polymyxa* that produces a large amount of polysaccharides possessing high activity against crown rot disease in peanut plants. Thus, the objectives of the present work were to investigate the modes of attachment and patterns of *P. polymyxa* colonization on peanut roots growing from inoculated seeds and on the surface of *A. niger* hyphae as well as evaluate the ability of *P. polymyxa* to induce systemic resistance in peanut plants.

MATERIALS AND METHODS

Strain and culture conditions

A. niger was isolated from roots and seeds of a diseased peanut field at Noubaria, Bohera governorate and maintained on Potato Dextrose agar. Fungal culture was identified in Plant Pathology Department, National Research Centre, Egypt. Fungus spores were washed from the agar with tap water containing 0.01% Tween-80 to prepare inoculum suspension. Inoculums concentrations were 1×10^5 spores ml^{-1}.

Two *P. polymyxa* strains B5 and B6 used in this study were isolated from the soil around peanut roots using Nutrient agar (NA), and identified according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986) based on its morphological and physiological characteristics. Isolates were grown in Nutrient broth at 30°C for 24 h. Cells were harvested by centrifugation (12,000 rpm, 10 min), washed twice, and then resuspended in sterile distilled water to a density of 10^6 bacteria ml^{-1}. For the production of polysaccharide, the bacteria were grown at 30°C with shaking (150 rpm) in a liquid medium containing 2% glucose, 0.3% bacto-peptone, 0.05% $MgSO_4$-$7H_2O$, 0.03% KH_2PO_4 and 0.07%K_2HPO_4. For long-term maintenance, strains were preserved in nutrient broth containing 15% (v/v) glycerol at -4°C.

In vitro antagonism test

Antagonistic activity was measured as zone inhibition on Potato Dextrose agar (PDA) and growth reductions through dual culture on solid PD or broth medium or filtrate inhibition, where volume of each of *B. polymyxa* culture filtrate was added to medium to provide a final concentration of 50%, then inoculated with equal discs of the tested pathogen. Colonies diameters or mycelium dry weight was determined after four days.

Measurements of bacteria cell growth, protein and biopolymers production

Cell growth, protein and biopolymers production of bacteria were measured in culture filtrates after four days of incubation. Cell growth was monitored by measuring the difference in protein contents between culture broth and culture supernatant. Cell growth was determined by measuring the optical density at 610 nm. Protein content in the supernatant was determined at 595 nm by the method of Bradford (1976) using bovine serum albumin as a standard. The amount of biopolymer was measured as dry weight after cells were removed from culture broth by centrifugation, and expressed as the weight (g/ml) of culture broth.

Biopolymers were purified from the culture broth by the method described by Kwon (1992). After the culture broth was diluted five times with distilled water it centrifuged at 20,000 rpm for 30 min, the supernatant was homogenized for 5 min at 10,000 rpm with a homogenizer, and then centrifuged again to remove the cells. The biopolymers in the supernatant were harvested as precipitants by ethanol precipitation and centrifugation, and then washed with 70% ethanol and re-dissolved in distilled water. The biopolymers were further purified by cetyl-trimethyl-ammonium bromide precipitation followed by ethanol precipitation again in 10% NaCl solution. After washing with 70% ethanol, the precipitated biopolymer were dried on a vacuum drier and dissolved in distilled water. The biopolymer were analyzed using infrared spectrum of the hydrolyzate and measured using a FT-IR (Fourier transform infrared) spectrophotometer with KBr pellets and a high performance liquid chromatograph (HPLC) equipped with a refractive index detector and an Aminex 87C column .

Inoculation of plants with *P. polymyxa*

P. polymyxa was used to protect peanut (Giza 4 cv.) against infection by *A. niger.* Seeds were surface sterilized using 0.3% (v/v) sodium hypochlorite for 10 to 12 min and then washed four times in

Table 1. Ability of *P. polymyxa* strains to inhibit mycelial growth of *A. niger.*

P. polymyxa strains	Zone of inhibition (mm)	Reduction in growth by 50% culture filtrate (%)	
		Linear growth (mm)	Dry weight (mg)
Control	0.0	90.0	40.1*
P. polymyxa (B5)	19.8	9.85	0.65
P. polymyxa (B6)	13.5	13.51	3.54
LSD	2.45	3.54	1.24

*Values represent the average of five replicates
LSD=Least significant difference at $P<0.05$.

sterile double distilled water. The seeds were then sown on water agar for germination test. Pots (30 cm diameter) were inoculated with *A. niger* at 2 x 10^4 cfu and 0.01% Tween-80. Peanut seeds were soaked in the *P. polymyxa* suspension at a rate of 10^6 cells/ml and sown directly 1 cm below the soil surface. Peanut were grown for 60 days in a greenhouse at temperature of 25°C. Ten replicates for the inoculated and control treatments were conducted. The soil water content of each pot was adjusted daily with sterile water, which was added onto the soil surface. The percentages of plant stand and crown rot disease infection were calculated after one and two months after sowing, respectively. Colonization of peanut roots rhizosphere and nonrhizosphere soil by *P. polymyxa* and *A. niger* was studied.

Scanning electron microscope

After one month of seeds sowing, roots and nonrhizosphere soil were collected and fixed in glutaraldehyde. The samples were dehydrated using a graded ethanol series and critical point dried in CO_2. The pressure was decreased very slowly to prevent tissue damage. Samples were examined by scanning electron microscope. All images were computer processed.

Enzymes assays

The chitinase activity was determined by colourimetric method of Boller and Mauch (1988). Colloidal chitin was used as substrate and dinitrosalicylic acid as reagent to measure reducing sugars. Chitinase activity was expressed as mM N-acetylglucose amine equivalent released/gram fresh weight tissue/60 min. β-1,3-Glucanase activity was assayed by colourimetric method of Nelson (1955). Reaction mixtures were incubated at 37°C for 30 min and were stopped by boiling for 5 min. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalents (expressed as glucose) per min .

Field experiments

Two successive field experiments were carried out during 2005 and 2006 seasons to evaluate the potentiality of *P. poelymyxa* in peanut plants against infection with *A. niger.* Trials were conducted at Experimental Farm of National Research Centre at El Kanater, Kalubaie governorate under natural infection conditions. A split-plot design with four replicates per treatment was used. Each individual plot consists of a five-row bed. Peanut seeds were sowed with 30 cm distance between plants and 50 cm between rows. Peanut seeds (Giza 4 cv.) were soaked in cell suspension of *P. polymyxa* at a rate of 10^6 cells mL^{-1} in a buffer containing 20 mM glucose and 20 mM potassium phosphate. One hundred seeds were used for each replicate. All the recommended methods of planting and watering were followed. No fertilizers were applied. The percentages of crown rot disease infection were calculated during different growth periods. Population densities of both *A. niger* (cfu x 10^4) and *P. polymyxa* (cfu x 10^7) isolates in rhizosphere and nonrhizosphere soil were scored monthly during the growth period. At harvest stage, pods and seeds contamination with *A. niger* (cfu x 10^3) were evaluated. Nodules dry weight; plant dry weight and yield of peanut plants were also agauged.

Statistical analysis

The collected data were statistically computed using the software SPSS for Windows (release 7.5.1, Dec. 20, 1996, SPSS Inc.). Data were subjected to analyses of variance and treatment means were compared by an approximate Duncan's multiple test ($P<0.05$).

RESULTS

In vitro antagonism test

In vitro, both strains of *P. polymyxa* were found to have significant inhibitory effects on *A. niger* (Table 1). When *P. polymyxa* applied as cells, significantly inhibited pathogen growth expressed as zone inhibition *P. polymyxa* (B5) displayed a high reduction in pathogen growth, which has large zone against *A. niger* compared to *P. polymyxa* (B6). The growth of *A. niger* was observed in the presence of culture filtrates (50%) from *P. polymyxa* in Petri plate and in liquid culture. Culture filtrate of the *P. polymyxa* strains clearly reduced growth of *A. niger* as linear growth on solid medium as well as dry weight in liquid medium. The highest reduction was observed by *P. polymyxa* (B5) followed by *P. polymyxa* (B6).

Growth, protein and biopolymer production by *P. polymyxa*

During the growth period of *P. polymyxa* in liquid medium, cell growth, protein and biopolymers concentrations were increased (Figure 1). The cell growth increased dramatically. Maximum level of cell growth was obtained after 72 and 96 h for *P. polymyxa* (B6) and *P. polymyxa* (B5), respectively and then decreased. The

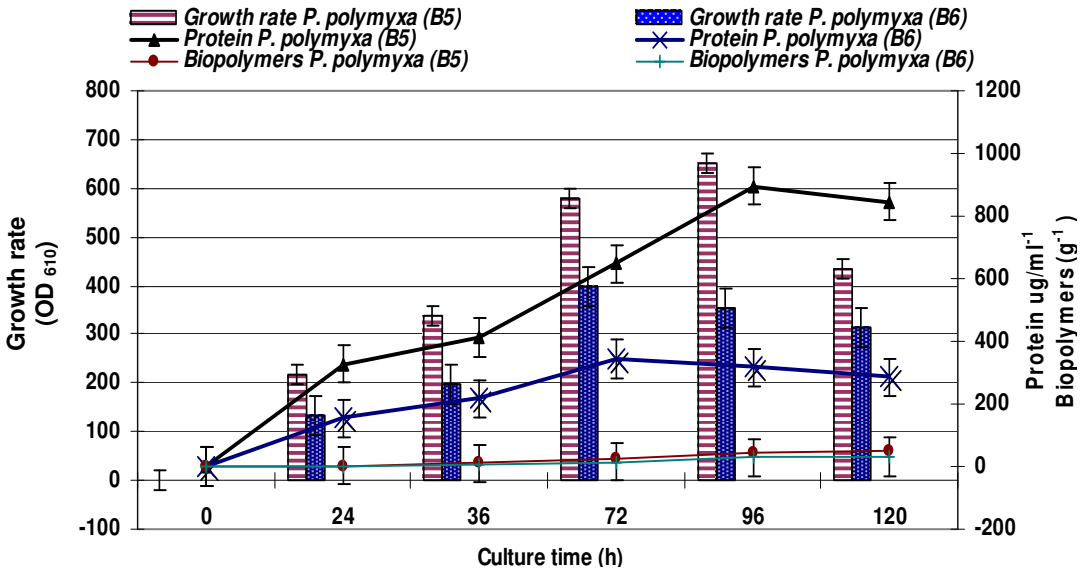

Figure 1. Cell growth, protein content and biopolymers production of *P. polymyxa* (B5) in liquid medium. During the culture, changes in cell growth, protein content and biopolymer concentration were monitored every 24 h. Cell growth was determined by measuring the optical density at 610 nm. Protein content was determined at 595.

Table 2. Ability of *P. polymyxa* strains to produce exopolysaccharides after four days of incubation in liquid medium.

***P. polymyxa* Strains**	**Polysaccharides (µM/ml^{-1})***			
	Glucose	**Galactose**	**Mannose**	**Xylose**
P. polymyxa (B5)	0.32	0.41	0.19	0.34
P. polymyxa (B6)	0.17	0.27	0.11	0.13

*Polysaccharides were analyzed using a high performance liquid chromatograph (HPLC) equipped with a reverse-phase HPLC of the *P. polymyxa* biopolymers with a refractive index detector and an Aminex 87C column.

total protein production increased very fast according to the increase of cell growth until the cell growth reached the maximum after 72 and 96 h, which was 343.8 and 894.6 µg/ ml $^{-1}$ of *P. polymyxa* (B6) and *P. polymyxa* (B5), respectively and then decreased dramatically thereafter. Changes in the biopolymers showed the same pattern as those in the protein concentration. Maximum level of biopolymers production was observed at 120 h of cultivation, which was 31.7 and 50 g/ml^{-1} biopolymers of *P. polymyxa* (B6) and *P. polymyxa* (B5), respectively in culture broth.

The FT-IR absorption spectrum of the biopolymers analysis of both strains of *P. polymyxa* showed stretches of OH and CH as well as groups of CHO, CH_2OH and peaks of C-O, which is a typical spectrum for carbohydrates. The peaks suggest that the polysaccharide is composed of a sugar derivative (data not shown). An HPLC analysis also showed similar pattern in the polysaccharide production by *P. polymyxa*, suggesting that the biopolymer is a homopolysaccharide, which consist of various sugars and sugar derivatives such as glucose, galactose, mannose and xylose (Table 2). *P. polymyxa* (B5) tend to produce high levels of galactose, xylose and glucose compared to the other strain.

Inoculation of plants with *P. polymyxa*

The competency of *P. polymyxa* on controlling crown rot disease of peanut under soils infested with *A. niger* is presented in Table 3. In control soil infested with *A. niger*, severe crown rot disease incidence of peanut was observed. Crown rot disease incidence highly decreases when the seeds were soak with the cells of *P. polymyxa* (B5). However, seeds were soaked with the cells of *P. polymyxa* (B6) resulted in a significant increase in plant stand and decrease in the crown rot disease incidence. Colonization of *A. niger* or *P. polymyxa* isolates in peanut rhizosphere and non rhizosphere was assessed throughout 60 days growth period. The propagates counts of *A.*

Table 3. Influence of seed treatment with *P. polymyxa* on crown rot disease incidence and population counts of *A. niger* of peanut plants grown under artificial infested conditions.

P. polymyxa Strains	Mean percent plant stand*	Crown rot incidence (%)	*A. niger* (cfu x 10^{-4})				*P. polymyxa* (cfu x 10^{-6})			
			Rhizosphere		Nonrhizosphere		Rhizosphere		Nonrhizosphere	
			30 d	60 d	30 d	60 d	30 d	60 d	30 d	60 d
Healthy check	93.5	6.87	---	---	---	---	---	---	---	---
Pathogen check	47.9	28.6	2.50	11.8	2.82	8 .66	---	---	---	---
P. polymyxa (B5)	99.4	0.0	---	---	---	---	4.85	13.6	2.87	10.7
P. polymyxa (B6)	99.5	0.0	---	---	---	---	3.97	11.6	2.54	9.76
P. polymyxa (B5)+ *A. niger*	99.5	0.75	0.50	0.35	2.98	1.34	3.36	10.5	2.67	9.56
P. polymyxa (B6)+ *A. niger*	98.6	1.06	1.45	0.95	6.87	2.45	3.01	9.45	2.45	9.45
LSD	0.23	0.45	0.65	0.54	0.98	0.87	0.33	0.67	0.23	0.31

*Mean percent plant stand was estimated one month after sowing. Crown rot incidence was estimated two months after sowing. LSD=Least significant difference at P<0.05.

Table 4. Chitinase and β-1,3-glucanase activities in peanut roots treated with *P. polymyxa* grown under artificial infested soil with *A. niger*.

Treatments	Chitinase[y] (unit)	β-1,3-glucanase[y] (unit)
Healthy check	5.75	19.9
Pathogen check	4.65	14.5
P. polymyxa (B5)	8.75	28.3
P. polymyxa (B6)	8.07	25.4
P. polymyxa (B5)+ *A. niger*	8.37	27.9
P. polymyxa (B6)+ *A. niger*	7.94	23.1
LSD	0.98	2.05

Chitinase and β-1,3-glucanase activities was estimated one month after sowing. Chitinase activity was expressed as mM N-acetylglucose amine equivalent released/g fresh weight tissue/60 min meanwhile, β-1,3-glucanase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalents (expressed as glucose) per min. LSD = Least significant difference at *P*<0.05

niger were higher in either rhizosphere or non rhizosphere of untreated plants after 30 and 60 days of sowing. *A. niger* propagates counts were non-detectable in the rhizosphere soil and non rhizosphere soil treated with the cells of *P. polymyxa* (B5). At the same time, there were two and three fold decreases of the *A. niger* populations on root rhizosphere or on non rhizosphere soil in the presence of *P. polymyxa* (B6). The ability of *P. polymyxa* to colonize the newly emerging roots was also measured. In sterile soil and inoculated seeds with bacteria, populations counts of both *P. polymyxa* strains were capable of increasing on either peanut roots rhizosphere or on non rhizosphere soil. *P. polymyxa* (B5) showed a significant increase in its population densities as compared to the strain (B6) in root rhizosphere or on non rhizosphere soil.

Scanning electron microscope

The ability of both *A. niger* or the promising *P. polymyxa* (B5) to colonize peanut rhizosphere and nonrhizosphere soil was assessed after one month of inoculation using scanning electron microscope (SEM) (Figures 2 and 3). In control soil infested with *A. niger*, strong proliferation of *A. niger* was observed on the roots (Figure. 2). The ability of *P. polymyxa* (B5) to colonize the roots grown in infested soil with *A. niger* was also measured. SEM indicated that the bacteria rapidly increased and colonized predominantly the root, where they formed biofilms compared with untreated control. At the same time, the rate and extent of pathogen colonization was markedly prevented in roots previously inoculated with *P. polymyxa*. Also, SEM revealed that colonization occurred preferentially in nonrhizosphere soil and colonized the hyphal of *A. niger* (Figure. 3).

Chitinase and β-1,3-glucanase assays

Results in Table (4) revealed that treatment of peanut seeds with *P. polymyxa* produced significant effect on levels of chitinase activity. Chitinase activity was greatly increased in peanut plants inoculated with *P. polymyxa*

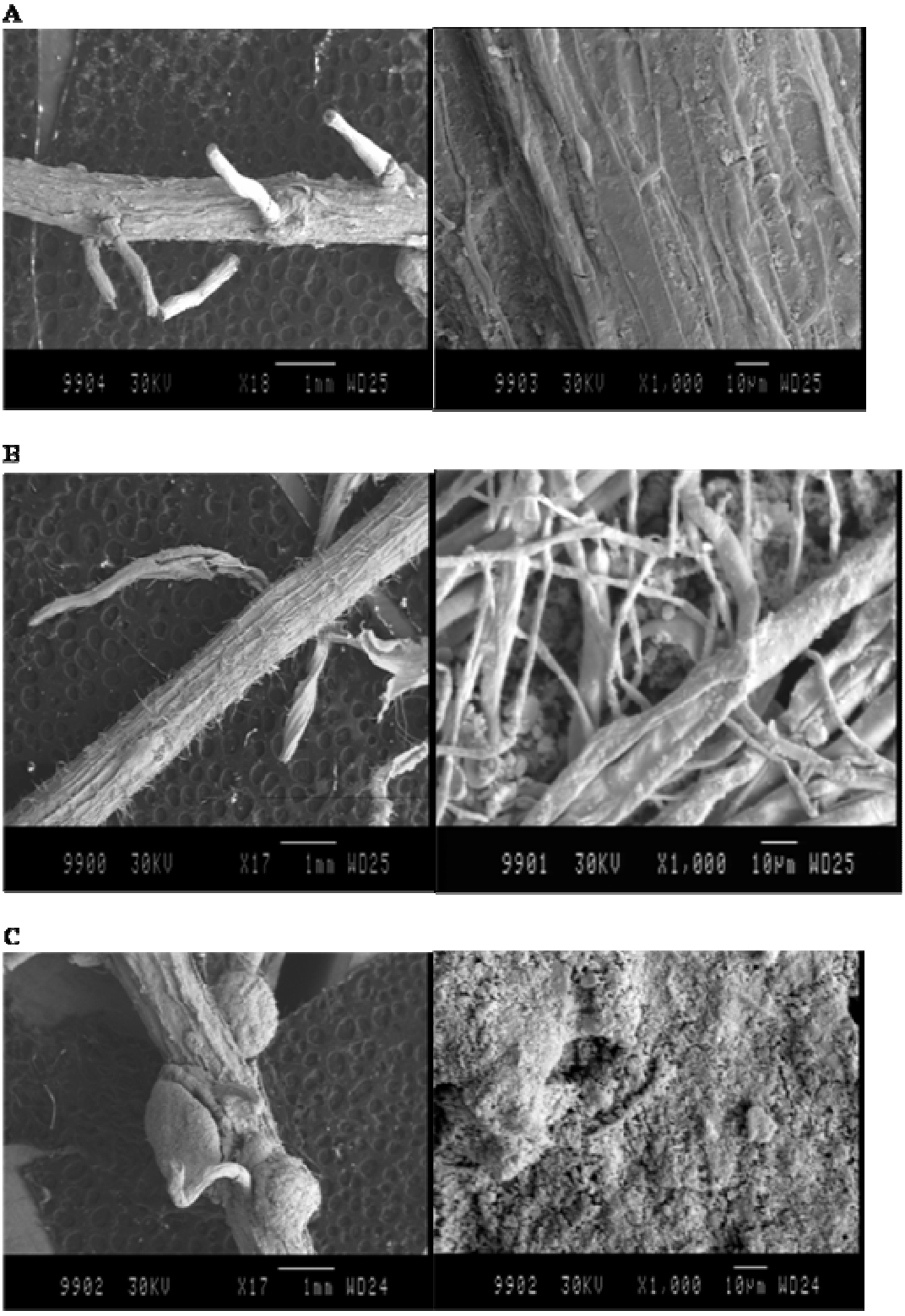

Figure 2. SEM micrographs of peanut roots colonized by *P. polymyxa* (B5). Untreated control (A); inoculated plants with *A. niger* (B) and roots of inoculated plants with *P. polymyxa* B5 and sown in infested soil with *A. niger* (C).

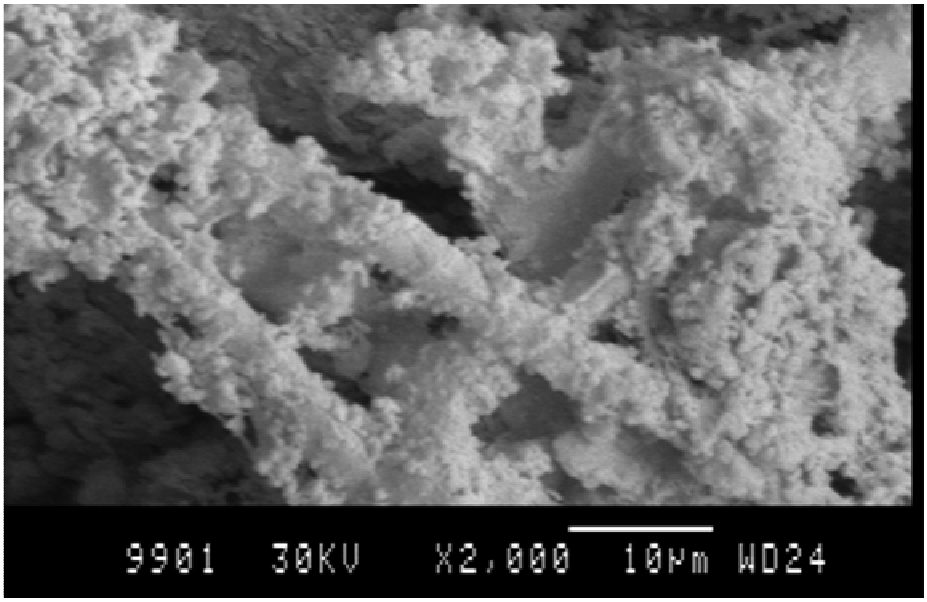

Figure 3. SEM micrographs of peanut nonrhizosphere soil by *P. polymyxa* (B5) showed that *P. polymyxa* B5 colonized *A. niger* in nonrhizosphere soil (D).

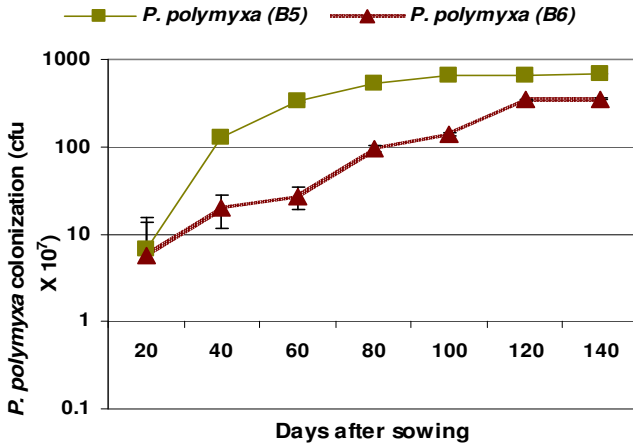

Figure 4. Population counts of *P. polymyxa* in the rhizosphere soil of peanut plants during growth periods after seed treatment and sown under field conditions. Data are means of 2005 and 2006 seasons.

compared with untreated control plants and grown in sterilized and infested soil with *A. niger*. Chitinase activity was greatly decreased in plants grown in soil inoculated with *A. niger* in comparison with untreated control. The greatest increase was obtained with *P. polymyxa* (B5). However, a high increase in chitinase activity was also obtained with *P. polymyxa* (B6). Data also showed that the pattern of β-1,3-glucanase activity was similar to that of chitinase activity. Since, activity of β-1,3-glucanase was greatly increased in treatment of *P. polymyxa* (B5) compared with untreated control and treatment of *P. polymyxa* (B6).

Field trials

The effect of *P. polymyxa* on roots colonization and controlling crown rot disease incidence of peanut was assessed throughout 140 days of growth period under natural infestation with *A. niger* (Table 5 and Figures 4 and 5). Data showed that crown rot disease incidence of peanut was higher in untreated plants after 140 days of sowing which reached 27.6 ± 1.3 and 29.7 ± 2.4% during 2005 and 2006 seasons (Table 5). Treated plants with *P. polymyxa* decreased the incidence of crown rot disease. *P. polymyxa* (B5) possessed considerably higher biocontrol activity, since a minimum level of disease symptoms was distinguished in those receiving nodules (B5), which reached to 0.2 6 ± 0.5 and 0.7 ± 0.4 % during 2005 and 2006 seasons, respectively.

Data also showed the effect of *P. polymyxa* on peanut nodules, plant growth and yield. Generally, the different growth parameters were higher in plants treated with *P. polymyxa* compared with untreated control during 2005 and 2006 seasons (Table 3). The increase in each of peanut nodules, plant growth and yield was significantly higher when associated with seeds treated with *P. polymyxa* (B5) than with *P. polymyxa* (B6) or untreated plants. This trend was true for different measurements during 2005 and 2006 seasons.

Population counts of *P. polymyxa* showed significantly greater increase in their population densities in soil rhizosphere through 140 days (Figure 4). Density of either *P. polymyxa* (B5) or *P. polymyxa* (B6) were highly increased from 6.76 and 5.76 cfu x 10^7 after 20 days of sowing to 682.8 and 356.0 cfu x 10^7 after 140 days, respectively. At the same time, *A. niger* propagates counts was decrea-

Table 5. Influence of seed treatment with *P. polymyxa* on crown rot disease incidence of *A. niger*, nodules dry weight, plant dry weight, and yield of peanut plants grown under field conditions.

P. polymyxa Strains	2005 season						2006 season					
	Crown rot incidence (%)	Nodules/plant		Plants dry weight (g)	Pods/plant (g)	Yield/plant (g)	Crown rot incidence (%)	Nodules/plant		Plants dry weight (g)	Pods/plant (g)	Yield/plant (g)
		Number	Weight (g)					Number	Weight (g)			
Control	27.6	86.8	1.87	16.7	65.9	47.8	29.7	81.8	1.45	15.8	63.1	46.8
P. polymyxa (B5)	0.2 6	326.8	4.65	33.6	98.4	86.6	0.7	306.2	4.22	32.5	95.2	82.8
P. polymyxa (B6)	2.4 6	276.8	4.12	29.6	93.6	80.5	2.9 6	254.5	3.95	29.5	90.2	86.8
LSD	0.54	38.5	0.85	3.05	4.21	5.87	0.96	33.6	0.76	2.85	4.56	3.67

*All experiments were repeated five times; LSD=Least significant difference at $P<0.05$.

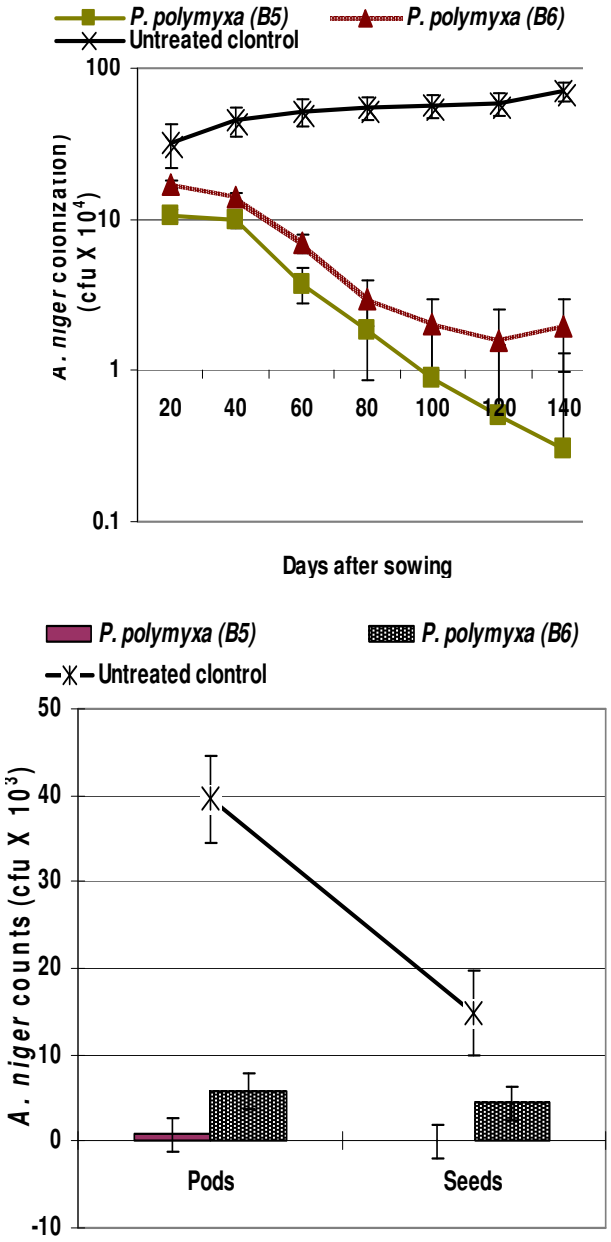

Figure 5. Influence of peanut seed treatment with *P. polymyxa* on rhizosphere soil, pods and seeds contamination with *A. niger* during growth periods under field conditions. Data are means of 2005 and 2006 seasons.

sed in the soil rhizosphere of plants treated with the *P. polymyxa* in comparison to untreated seeds (Figure 5). A minimum propagates were recorded in the soil rhizosphere of peanut plants inoculated with *P. polymyxa* (B5) reached to 0.3 cfu x 10^4 compared with 69.7 cfu x 10^4 of untreated control after 140 days of sowing.

Pods and seeds contamination with *A. niger* were evaluated at harvest during 2005 and 2006 seasons (Figure 5). Pods and seeds contamination with *A. niger* was higher in untreated plants. It was observed that pods and seeds significantly had lower counts of *A. niger* in peanut plants inoculated with *P. polymyxa*. *P. polymyxa* (B5) inhibited seed contamination with *A. niger*.

DISCUSSION

The use of microorganisms to control plant diseases offers an attractive alternative to the use of synthetic chemicals (Roberts et al., 2005). The abundance of a beneficial strain of microorganism in the vicinity of plant roots may suppress plant pathogens without producing lasting effects on the rest of the soil microbial and plant communities. Two natural strains of *P. polymyxa*, B5 and B6 were chosen as candidates for localization studies. *P. polymyxa* strains were chosen because this specie is gaining recognition for biocontrol in a variety of plants, even though it is mainly use as a seed protectant and antifungal agent (Timmusk et al., 2003). When we tested the biocontrol efficiency of *P. polymyxa* against crown root infection caused by *A. niger*, there was reduced pathogen growth in solid and liquid culture. Our findings also showed that *P. polymyxa* produce biopolymers in culture filtrate. Chemical analysis of polymers using infrared spectroscopy, and high performance liquid chromatography revealed that the biopolymer is a homopolysaccharide. Polysaccharides secretion is important in *P. polymyxa* biofilm development. Development of biofilm is closely associated with the generation of matrix, the majority of which is extracellular material.

Also, establishing a clearer understanding of *P. polymyxa* mechanism of perpendicular attachment to plant and hyphal surfaces may be important in realizing its potential as a biocontrol agent. Inoculation of peanut seeds with *P. polymyxa* led to pronounced colonization of the rhizosphere of peanut and of soil by these strains. Colonization is initiated by bacterial accumulation on the roots that occurred in the form of a biofilm and protected root from crown rot infection as we observed perpendicular attachment of this bacterium to both plant and hyphal cell walls. Biofilm formation accompanying this perpendicular mode of attachment in theory could support a larger population of bacteria on a given plant surface. The fact that in non-sterile soil experiments a *P. polymyxa* biofilm hindered indigenous bacterial colonization around the roots indicates that this *P. polymyxa* isolate could be a potentially aggressive biocontrol agent for use against pathogens which colonize the root. In addition, we observed markedly reduced pathogen colony formation on peanut rhizosphere and nonrhizosphere compared with the untreated seeds. *P. polymyxa* has also been isolated from the internal root tissues of white clover, perennial ryegrass, crested wheatgrass (Holl et al., 1988) and green bean (Petersen et al., 1996) cucumber (Roberts et al., 2005) and garlic (Kajimura and Kaneda, 1996) and has been shown to colonize seedlings systemically (Van Peer and Schippers, 1992). Biofilm formation is a major bacterial adaptive strategy to environmental conditions in aquatic and other settings (Davey and O'toole, 2000).

The observations reported here also show that *P. polymyxa* has characteristics of PGPR. Inoculation with this root-colonizing bacterium conferred partial resistance to crown rot disease. The induction level of protein was high in treated roots than in untreated plants. This may suggest that the protein plays a main function in the defense against biotic stress, which initiated a systemic response that resulted in partial protection from the pathogen upon subsequent challenge. Moreover, results in this study showed stimulation of the activities of chitinases and β-1,3-glucanase in inoculated plants with *P. polymyxa*. Increase in chitinases and β-1,3-glucanase in plants has known correlation with resistance to pathogens (Timmusk et al., 1999). Lipopolysaccharides from bacterial outer membranes have been proposed to be involved in the induction of induce systemic reaction (Van Peer and Schippers, 1992). The production of EPS possibly enhances water retention in the microbial environment and seems to regulate the diffusion of carbon sources such as glucose (Amellal et al., 1998**)** as well as stabilization**.** A further possibility is that *P. polymyxa* treatment results in increase plant stand, growth and yield. The results indicate that inoculation by the PGPR *P. polymyxa* can protect peanut against a fungal pathogen due to a combination of antibiosis, induction of plant resistance and characteristic exopolysaccharide production at the rhizoplane of the host plant.

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