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Induction of defense responses in soybean plants against macrophomina phaseolina by some strains of plant growth promoting rhizobacteria

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ABSTRACT

In recent years, interest in the ability of beneficial microorganisms to induce resistance in plants has grown, particularly with respect to their use as environmentally safe controllers of plant diseases. In the present study, pots and field experiments were conducted in Ismailia, Agric. Res. Station to evaluate the impact of some strains of plant growth promoting rhizobacteria (PGPR), individual or in mixed, (Bradyrhizobium japonicum strain USDA 110, Azotobacter chroococcum, Azospirillum brasilense, Bacillus megaterium, B. cereus and Pseudomonas fluorescens) for their ability to induce pathogenesis-related (PR)-proteins (chitinase and β-1,3-glucanase), peroxidase, phenylalanine ammonia-lyase (PAL) and phenolics against Macrophomina phaseolina (Mph) on soybean plants. In addition, populations of culturable bacteria and fungi in the rhizosphere soybean plants were also examined since inoculation with these microorganisms can affect both quantitatively and qualitatively the communities in the plant rhizosphere. PGPR-treated plants were effective in reducing diseases produced by Mph infection and evidence points to a combination of local and systemic mechanism being responsible for this bioprotector effect. Soil application with PGPR significantly increased the activity of peroxidase, chitinase, β-1, 3-glucanase and PAL and the accumulation phenolics in soybean plants compared to untreated control. When the induced plants were inoculated with Mph, several fold increase in the accumulation of phenolics and activities of defense enzymes was observed. The mixed inoculation did not give greater protection than single inoculation. On the other hands, microbial inoculation modifications of the microbial community structure and ecology were found. These results suggest that enhanced activities of defense enzymes and elevated content of phenolics may contribute to protection of soybean plants against Macrophomina phaseolina (Mph).

Key words: Macrophomina phaseolina, plant growth promoting rhizobacteria, induce resistance, soybean.

Introduction

Induced protection of plants against various pathogens by biotic or abiotic agents has been reported since 1930s when Chester (1933) proposed the term \textquoteleft acquired physiological immunity. Since then several terms have been used to describe the phenomenon of induced resistance such as \textquoteleft systemic acquired resistance (Ross, 1961), translocated resistance (Hurbert and Helton, 1967) and \textquoteleft plant immunization (Tuzun and Kuc, 1991). Plant growth promoting rhizobacteria (PGPR) are root colonizing bacteria with beneficial effects including plant growth promotion and biological disease control. In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under pots and field conditions (Viswanathan, 1999; Viswanathan and Samiyappan, 1999; Attia and Hamed, 2005). Other important mechanisms include production of lytic enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Attia and Hamed, 2005), HCN production (Defago et al., 1990) and degradation of toxin produced by pathogen (Duffy and Defago, 1997). In addition, competition for nutrients and space (Elad and Chet, 1987), antibiosis by producing antibiotics viz., pyrrolnitrin, pyocyanine, 2,4-diacyl phloroglucinol (Pierson and Thomashow, 1992) and production of siderophores (fluorescent yellow green pigment), viz., pseudobactin which limits the availability of iron necessary for the growth of pathogens (Kloepper et al., 1980; Lemanceau et al., 1992). The utilization of natural PGPR strains as inducers of plant defense responses may increase the chance of their applicability and/or a practical way to deliver immunization. PGPR strains applied as a seed-treatment resulted in a significant reduction in anthracnose disease caused by Colletotrichum orbiculare in cucumber (Wei et al., 1991, 1996). Similarly, induction of systemic resistance by Pseudomonas putida strain 89B-27 and Serratia marcescens strain 90-166 reduced Fusarium wilt of cucumber incited by Fusarium oxysporum f.sp. cucumber (Liu et al., 1995). PGPR as a seed-treatment alone or as seed-treatment plus soil drenching has protected cucumber plants against anthracnose disease (Wei et al.,

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In rice seed-treatment followed by root-dipping and a foliar spray with P. fluorescens strains Pf1 and FP7 showed higher induction of ISR against the sheath blight pathogen, Rhizoctonia solani (Vidyasekaran and Muthamilan, 1999). Similarly, in sugarcane, Viswanathan and Samiyappan (1999) established PGPR-mediated ISR against Colletotrichum falcatum causing red rot disease. PGPR can also induce systemic protection against bacterial diseases. Seed treated with P. fluorescens strain 97 protected beans against halo blight disease caused by Pseudomonas syringae pv. phaseolicola (Alstrom, 1991), while treatment of cucumber seed with P. putida strain 89B-27 and S. marcescens strain 90-166 decreased the incidence of bacterial wilt disease (Kloepper et al., 1993). These experiments show that PGPR strains initiate ISR against a wide array of plant pathogens causing fungal, bacterial and viral diseases.

Recently, we found that PGPR were the positive impact in improving the stand and vigour of soybean plants in Macrophomina infested soil under pot and field experiments and significantly decreased damping-off, rotted and wilted plants and increased healthy plants compared with the infested soil (El-Barougy et al, 2009).

In the present study, we evaluated the ability used of nitrogen fixing bacteria (Bradyrhizobium, Azotobacter, Azospirillum) in combination with Bacillus and Pseudomonas to induction of defense mechanisms related enzymes and accumulation of phenols in soybean leaves. In addition, influence of different microbial inoculants upon ecosystem processes such as bacterial populations in rhizosphere soil.

Materials and methods

Microorganisms used, inoculums preparation and inoculation techniques Bacterial (Plant growth promoting rhizobacteria):

Highly efficient strains of plant growth promoting rhizobacteria (PGPR) (Bradyrhizobium japonicum strain USDA 110, Azotobacter chroococcum, Azospirillum brasilense, Bacillus megaterium, B. cereus and Pseudomonas fluorescens) were isolated previously from rhizoplane and rhizospheric soil fractions of the soybean cultivars grown on a infested soil (El-Barougy et al, 2009) and identified as described earlier. The growth promoting rhizobacteria were independently grown in nutrient broth for 48 hours at 30°C in (Turky et al., 2007) a rotary shaking incubator. The PGPR producing bacteria inoculum was prepared in flasks and each flask containing 60 mL broth was inoculated with selected strains of bacteria and incubated for 72 hours under shaking (100 rpm) conditions at 28 ± 1°C. An optical density of 0.5 measured at a wavelength of 535 nm was achieved by dilution to maintain uniform cell density (10^-10^ CFU mL^-1) prior to seed inoculation. In PGPR treatments, 10 ml of either tested microorganisms suspension were added to the soil in each pot just after sowing. In field inoculation, seeds of soybean were inoculated with peat mixed with 10% sugar solution (inoculum to peat ratio 1:1 w/w).

Pathogenic (Macrophomina phaseolina):

M. phaseolina was originally isolated on potato dextrose agar (PDA) medium from diseased soybean plants collected from different localities of Ismailia Governorate (El-Barougy et al, 2009). Purification of the isolated fungus was carried out using hyphal tip techniques and identified according to their morphological characters according Willey (1976). The pathogenicity test of M. phaseolina was carried out at Ismailia Agric. Res. Station., on soybean (Kilarce) in pot infested soil using the homogenized culture technique. Inoculum of M. phaseolina was prepared in laboratory by multiplying the pathogen on oat (Avena sativa) grains. The grains (150 g) were soaked in distilled water overnight, autoclaved twice (121°C for 1 h) and inoculated with one agar disc (5 mm diameter) cut from the margin of an actively growing 5-day-old pathogen culture. Flasks were incubated at 28±1°C in the dark for 15 days. Oat seed containing mycelial fragments plus sclerotia served as inoculum. The population of M. phaseolina was recorded as 1.2×10^7 colony-forming units (CFUs) per oat seed by dilution plate technique using chloroneb-rose Bengal-mercuric chloride- agar medium (Filho and Dhingra 1980).

Pots and field experiments:

Soybean seeds Giza 35 (10 seeds/pot) were sown in 30-cm pots filled with unsterilized M. phaseolina infested soil at the rate 2.5 g oat seeds mixed properly per pot as previously mentioned, one day before planting. All plants were inoculated with nitrogen fixing bacteria (NFB) Bradyrhizobium japonicum strain soil infested with USDA 110, Azotobacter chroococcum and Azospirillum brasilense (NFB). NFB inoculation was done by 10 ml per pot. The treatments were as follows: plant inoculated with (NFB) plus B. megaterium, or B. cereus, or Pseudomonas fluorescens and mixture of PGPR. The control treatments were soil infested with M. phaseolina
was used as control 1 and uninfested soil as control 2. A set of 4 pots for each treatment were used. Each pot received equal amounts of water. Other agricultural processor was performed according to normal practice.

Experimental design field trials were conducted at Ismailia Agric. Res. Station in naturally infested field. The same treatments in pot experiment were used arranged in a complete randomized block designed with four replicates. The field plot was 3x3 m with 5 rows, 200 seeds were sown in each plot (40 seed row\(^{-1}\)). For both trials fertilization was applied throughout the season by drip irrigation.

**Sampling:**

Plant samples were taken at 15 and 30 days after planting, respectively to observations the percentages of pre and post emergence damping off. Healthy and infected survival plants were evaluated and discoloration of internal tissue was recorded three months after seeding. To determine microbiological analyses, three plants per plot were collected at 7, 14, 21 and 35 days. Plants were individually placed in plastic bags and transported in a cooler to the laboratory for immediate processing.

**Disease severity of root rots and wilt disease complex:**

Incidence (%) of diseased plants (Total number of dead plants/Total number of plants at plots (%)) was calculated 2 and 3 months after planting. Disease severity was also recorded on a random sample of plants of the plots (20 plants) four months after planting. Disease severity indexing (DSI) of root rot and any discoloration of tissue were recorded according to based on 0-4 scale according percentage of foliage yellowing or necrosis (Haware and Nene, 1980) based on (0=0%, 1=1-33%, 2=34-66%, 3=67-100%, 4= dead plant).

**Evaluation of total culturable bacteria, total fungi, Pseudomonas and Bacillus in the rhizosphere soil of soybean plants:**

Rhizosphere samples were obtained by collecting the soil adhering to the roots. The 10 g of soil samples were placed in an Erlenmeyer flask containing 90 ml of sterilized distilled water, and shaken for 30 min. Ten-fold series dilutions were prepared, and appropriate dilutions were plated in specific media. The numbers of colony forming bacterial cells and total saprotrophic fungi were determined in PDA medium by plate dilution (Johnson and Curl, 1972). For the quantification of bacterial cfu, cycloheximide (100mg ml\(^{-1}\)) was added to the growing medium. For the quantification of *Pseudomonas*, King's medium was used. Samples were then heated to 80 °C for 20 min and the -3 and -4 dilutions were plated on 10% TSA to assess *Bacillus*.

**Induction of defense mechanism with PGPR:**

**Protein extraction:**

Seedlings were divided into portions, stems and roots were excluded, and leaves were separated, washed under running tap water for 5 min, dried gently, weighed, and ground with a mortar and pestle. The ground matter was homogenized (2 min, 4°C) in phosphate buffer (1:2 w/v, pH 6, 0.05 M) by use of Corex tubes. The homogenate was centrifuged twice at 10,000 \(g\) and 4°C, and the supernatant was collected and kept at -20°C was used in the enzyme assay.

**Detection of chitinase:**

The total chitinase activity assay was based on the colorimetric determination of \(p\)-nitrophenyl cleaved from a chitin-analogous substrate, \(p\)-nitrophenyl-\(β\)-D-\(N\),\(N\)′-diacetylchitobiose (PNP) (Roberts and Selitrennikoff 1988). A crude enzyme preparation and 10 µl of PNP stock solution (2 mg/ml) were added to 50 mM acetate buffer (pH 5.0) to a total volume of 0.5 ml and incubated for 2h in a water bath at 37°C. The reaction was terminated with 0.5 ml of 0.2 M Na\(_2\)CO\(_3\). An extinction coefficient of 7 X 10\(^3\) mM\(^{-1}\) cm\(^{-1}\) at 410 nm was used to determine \(p\)-nitrophenyl release from the substrate. Chitinase activity was expressed as millimoles of PNP produced per gram of fresh tissue per hour.

**Detection of Peroxidase activity:**

Peroxidase activity was assayed spectrophotometrically at 610 nm with phenol red as a substrate. The complete reaction mixture (1 ml, 37°C) contained 10 to 20 µl of a crude enzyme preparation, 50 µl of 0.2% (wt/vol) phenol red, and 50 mM sodium citrate (pH 4.2). Reactions were initiated with 10 µl of 1 mM hydrogen peroxidase.
peroxide and stopped after 3 min with 40 µl of 2 N sodium hydroxide. The optical density was detected at 610 nm as described above. The absorbance was recorded at 610 nm and calculated with a molar extinction coefficient of 122,000 M⁻¹ cm for the oxidized product (Ruttimann et al., 1992). Peroxidase activity was expressed as millimoles of phenol red oxidized per gram of fresh tissue per minute.

Detection of β-1, 3-glucanase activity:

β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan et al., 1991). One-gram leaf samples were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C using pre-chilled pestle and mortar. The extract was then centrifuged at 10,000 g for 15 min at 4°C and the supernatant was used as enzyme source. The reaction mixture consisted of 62.5 ml of laminarin (4%) and 62.5 ml of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was stopped by adding 375 ml of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. The blank consisted of crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as mg of glucose equivalents min⁻¹ g⁻¹ fresh tissue.

Detection of phenylalanine ammonialyase (PAL) activity:

PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm as described by Dickerson et al. (1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. In reference cell, 0.4 ml of enzyme extract was taken along with 1 ml of borate buffer. The amount of transcinnamic acid synthesized was calculated as described by (Dickerson et al., 1984). Enzyme activity was expressed on a fresh weight basis (nmole of transcinnamic acid min⁻¹ g⁻¹).

Detection of phenolic content:

Soybean leaves (1 g) were homogenized in 10 ml of 80 percent methanol and agitated for 15 min at 70°C (Swain and Hills, 1959). One ml of the methanolic extract was added to 5 ml of distilled water and 250 ml of Folin Ciocalteau reagent (1 M) and the solution was kept at 25°C. After 3 min 1 ml of saturated solution of Na₂CO₃ and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue colour was measured using a spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with phenol and expressed as phenol equivalents in mg g⁻¹ fresh weight.

Statistical analysis:

Data were statistically analyzed according to standard procedures for analysis of variance (general linear model) and mean separation (least significant difference) (SAS Institute, Cary, NC). All differences referred to in the text were significant at the 5% level of probability.

Results:

1-Pots experiment:

Data in Table (1) show that all bacterial plant growth promoting decreased damping off and percentage of infested plants and increased healthy plants compared with the control treatment. Data indicate that soil infested with M. phaseolina used as a control showed the highest percentage of pre emergence damping off (25%) compared with the lowest percentage (5%) recorded from non-infested soil followed by soil artificially infested and treated with B. megaterium (20%) while, 12.5% obtained from mixed inoculation. For post emergence damping off, the soil infested with M. phaseolina recorded the highest percentage of infected plants (20%) but the lowest percentage which reached 2.5% was obtained from both of B. cereus and Pseudomonas fluorescens treatments. Concerning the percentage of healthy plants, the most effective treatment was B. megaterium at (75%); followed by B. cereus showed 72.5% while Pseudomonas fluorescens showed 62.5 % healthy survival compared with the lowest percentage (37.5%) recorded from infested soil. Root-rot severity was 3.6 when M. phaseolina was applied alone and severity was reduced to 2.3, 1.8, 1.6 and 1.3 in plants grown from seed treated with Pseudomonas fluorescens, B. cereus, mixed inoculation and B. megaterium respectively, when sown in
soil infested with *M. phaseolina* comparing with the value of 0.7 which recorded from plants grown in non-infested soil.

2-Field experiment:

**Total saprotrophic fungi and bacteria populations:**

In generally, dually inoculation of PGPR was greater populations of bacteria (cfu/g dry soil weight) in comparison with the uninoculated treatments (Table2). Populations of total culturable bacteria decreased in *Bacillus* inoculated rhizospheres under pots and field experiments compared to Ps and mixed inoculation. Occasional significant decreases were observed in the number of total fungi isolated from PGPR treatments compared to untreated treatments although; this effect was less significant in field experiment (Table 2).

**Table 1:** Effect of some plant growth promoting rhizobacteria (PGPR) on damping off and survival plants of soybean seedling infected with *Macrophomina phaseolina*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-emergence</th>
<th>Post-emergence</th>
<th>Infested survival</th>
<th>Healthy survival</th>
<th>Disease severity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFB</td>
<td>5.0c</td>
<td>0.0c</td>
<td>0.0c</td>
<td>95.0a</td>
<td>0.7d</td>
</tr>
<tr>
<td>NFB+M ph</td>
<td>25.0a</td>
<td>20.0a</td>
<td>17.5a</td>
<td>37.5c</td>
<td>3.6a</td>
</tr>
<tr>
<td>NFB+BM</td>
<td>20.0ab</td>
<td>2.5bc</td>
<td>2.5b</td>
<td>75.0ab</td>
<td>1.3cd</td>
</tr>
<tr>
<td>NFB+BC</td>
<td>12.5bc</td>
<td>12.5ab</td>
<td>2.5b</td>
<td>72.5ab</td>
<td>1.8bc</td>
</tr>
<tr>
<td>NFB+PF</td>
<td>12.5ab</td>
<td>2.5bc</td>
<td>0.0c</td>
<td>62.5bc</td>
<td>2.3b</td>
</tr>
<tr>
<td>PGPR mixed</td>
<td>12.5bc</td>
<td>5.0bc</td>
<td>15.0a</td>
<td>67.0ab</td>
<td>1.6c</td>
</tr>
</tbody>
</table>

Figures in the same column followed by the same letters are not significantly different (p> 0.05) based on Duncan’s multiple range test. NFB= Nitrogen fixing bacteria, M ph= *Macrophomina phaseolina*, BM= *Bacillus megaterium*, BC= *B. cereus*, PF= *Pseudomonas fluorescens*.

**Table 2:** Effects of inoculation with PGPR on survival of indigenous rhizosphere microorganisms in pot and field trials in rhizosphere soybean plants.

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>Total bacteria (10⁶ cfu/g dry soil)</th>
<th>Total fungi (10⁴ cfu/g dry soil)</th>
<th>Pseudomonas spp (10⁵ cfu/g dry soil)</th>
<th>Bacillus spp. (10⁵ cfu/g dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pot</td>
<td>Field</td>
<td>Pot</td>
<td>Field</td>
</tr>
<tr>
<td>At 7 days after planting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFB</td>
<td>47.2d</td>
<td>62.5b</td>
<td>5.0b</td>
<td>9.2b</td>
</tr>
<tr>
<td>NFB+M ph</td>
<td>48.2d</td>
<td>62.0b</td>
<td>15.3a</td>
<td>13.5a</td>
</tr>
<tr>
<td>NFB+BM</td>
<td>54.6c</td>
<td>71.4a</td>
<td>2.0cd</td>
<td>6.3d</td>
</tr>
<tr>
<td>NFB+BC</td>
<td>56.9bc</td>
<td>72.6a</td>
<td>3.2bc</td>
<td>7.7c</td>
</tr>
<tr>
<td>NFB+PF</td>
<td>58.0ab</td>
<td>73.0a</td>
<td>2.9c</td>
<td>6.3d</td>
</tr>
<tr>
<td>PGPR mixed</td>
<td>60.0a</td>
<td>73.5a</td>
<td>2.1d</td>
<td>8.2bc</td>
</tr>
</tbody>
</table>

At 21 days after planting

| NFB                  | 63.0c    | 83.5d          | 6.6b     | 11.5b          | 9.2c    | 11.5c          | 5.3e    | 5.2d           |
| NFB+M ph             | 67.0c    | 84.0d          | 19.4a    | 23.1a          | 9.3c    | 12.8c          | 7.8de   | 9.1e           |
| NFB+BM               | 88.1a    | 106.5b         | 3.8c     | 3.9c           | 9.2c    | 17.7b          | 25.4b   | 33.7b          |
| NFB+BC               | 78.6     | 93.9c          | 2.8c     | 3.8c           | 9.6c    | 15.4bc         | 28.4a   | 37.9a          |
| NFB+PF               | 67.6c    | 94.8c          | 2.7c     | 3.5c           | 28.8b   | 25.9a          | 9.4cd   | 9.2c           |
| PGPR mixed           | 70.6b    | 119.8a         | 3.8c     | 3.7c           | 30.1a   | 27.6a          | 26.0ab  | 36.1a          |

At 35 days after planting

| NFB                  | 57.1d    | 76.7a          | 3.42a    | 4.7b           | 7.8d    | 10.9c          | 5.73a   | 6.3d           |
| NFB+M ph             | 55.5d    | 75.5d          | 2.57a    | 13.3a          | 16.2c   | 11.4bc         | 5.29a   | 6.0d           |
| NFB+BM               | 76.9a    | 96.0b          | 3.31a    | 4.8b           | 8.3d    | 12.74b         | 5.79a   | 16.3b          |
| NFB+BC               | 67.5c    | 96.6b          | 3.13a    | 4.5b           | 8.2d    | 12.66b         | 5.64a   | 11.8c          |
| NFB+PF               | 55.4d    | 85.8c          | 2.54a    | 4.4b           | 21.4a   | 16.55a         | 6.12a   | 6.7d           |
| PGPR mixed           | 73.2b    | 102.1a         | 2.58a    | 4.2b           | 19.1b   | 15.40a         | 6.32a   | 20.2a          |

At 120 days (End of season)

| NFB                  | 62.1a    | 72.5a          | 8.27a    | 11.40a         | 11.90b  | 12.72a         | 4.98a   | 5.4c           |
| NFB+M ph             | 62.5a    | 73.6a          | 6.31b    | 8.98b          | 19.20a  | 12.89a         | 4.97a   | 5.1c           |
| NFB+BM               | 62.9a    | 72.5a          | 7.50a    | 7.53bc         | 14.60   | 12.86a         | 5.11a   | 15.6a          |
| NFB+BC               | 62.9a    | 72.8a          | 6.57b    | 7.09c          | 10.01b  | 12.86a         | 5.23a   | 11.5b          |
| NFB+PF               | 61.8a    | 73.1a          | 6.37b    | 7.18c          | 10.71b  | 12.94a         | 5.27a   | 5.7c           |
| PGPR mixed           | 61.8a    | 71.6a          | 7.30ab   | 7.22c          | 10.09b  | 12.34a         | 5.34a   | 15.2a          |

Figures in the same column followed by the same letters are not significantly different (p> 0.05) based on Duncan’s multiple range test. NFB= Nitrogen fixing bacteria, M ph= *Macrophomina phaseolina*, BM= *Bacillus megaterium*, BC= *B. cereus*, PF= *Pseudomonas fluorescens*. 

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In both the pots and field trials at day after plating (DAP), the applied strains were detected in treated plots but were detected at significantly lower levels in untreated plots (Table 2). Treatments had a small significant effect on the number of total heat tolerant bacteria isolated but did not influence total overall bacteria, indicating a shift in the composition of these communities (Table 2). There was largely no effect of added PGPR on numbers of naturally occurring beneficial bacteria including fluorescent pseudomonades (Table 2). For both studies, similar trends were observed at 21 DAP, and 35 DAP except with a more pronounced increase of total heat tolerant bacteria and total bacteria isolated from PGPR treatments when compared to the untreated control (Tables 2). In addition, at 35 DAP there was a significant increase in fluorescent pseudomonades in all PGPR treatments when compared to the untreated control for both trials (Table 2). By harvest time at 120 DAP few differences were evident in indigenous populations with the exception of an increase in fungi populations with most treatments, while the number of the applied strains remained consistently high in the field trial (Table 2).

The incidence of disease was significantly lower in PGPR-treated soybean plants than in uninoculated treatment (Table 3). Maximum levels of pathogen inside the roots were detected in non-inoculated plants with PGPR, followed by those in dually inoculation and the minimum level of the pathogen was detected in plants inoculated with in mixed inoculation with PGPR.

**Table 3:** Effect of bacterial plant growth promoting and bacterial biocontrol agents alone or in combination on root rot and wilt diseases complex of soybean plant under field condition.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>One months after sowing</th>
<th>Two months after sowing</th>
<th>Three months after sowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease Incidence (%)</td>
<td>Reduction over control</td>
<td>Disease Incidence (%)</td>
</tr>
<tr>
<td>NFB</td>
<td>65.0a</td>
<td>-</td>
<td>85.0a</td>
</tr>
<tr>
<td>NFB + BM</td>
<td>24.0bcd</td>
<td>63.07</td>
<td>20.0ef</td>
</tr>
<tr>
<td>NFB + BC</td>
<td>16.0cde</td>
<td>75.30</td>
<td>67.5b</td>
</tr>
<tr>
<td>NFB + PF</td>
<td>27.3b</td>
<td>58.00</td>
<td>55.0c</td>
</tr>
<tr>
<td>PGPR Mixed</td>
<td>43 b</td>
<td>33.80</td>
<td>50.0c</td>
</tr>
</tbody>
</table>

Figures in the same column followed by the same letters are not significantly different (p> 0.05) based on Duncan’s multiple range test. NFB=Nitrogen fixing bacteria, M ph= Macrophomina phaseolina, BM= Bacillus megaterium, BC= B. cereus, PF= Pseudomonas fluorescens

**Activation of defense mechanisms:**

These activities in leaves were measured at 3, 6, 9, and 15 days post inoculation. The results in Fig (1) indicated that soil inoculation with PGPR and/or *M. phaseolina* resulted in significant increase in the activities of chitinase and peroxidase in leaves of soybean plants (Fig. 1). Chitinase activity peaked at 9 days in leaves of inoculated plants, while in noninoculated plants, activity increased gradually with time. At peak chitinase activity, inoculated plants showed a 3.2-fold increase compared with uninoculated plants. The peroxidase activity increased in leaves of soybean plants due to antagonists treatments and also due to inoculation with pathogen. The increase has lasted up to 6 days after inoculation with PGPR, while uninoculated plants showed a gradual increase with time. The maximum activity of the enzyme was observed in *Bacillus* inoculated plants followed by *Pseudomonas* inoculated plants. When compared to control 2- to 3-fold increase in peroxidase activity was noticed due to inoculated with antagonists and/or pathogen inoculation (Fig 1). From 9 to 15 days, a two-to threefold decrease in both activities in the leaves of treated plants was observed. In the present study, it was observed that application of antagonists and inoculation with pathogen triggered the activity of β-1,3-glucansase, PAL and the accumulation of phenolic content in leaves of soybean plants. The maximum PAL activity and total phenolic content were observed in *Bacillus* inoculated plants followed by *Pseudomonas* inoculated plants. The increase of antagonists treated plants (Fig 1). Inoculation with *M. phaseolina* in the antagonists treated plants the activity of β-1,3-glucansase and PAL increased several fold 6 days after treatment, respectively and then decreased gradually. The increased PAL activity and accumulation of phenolics in the biocontrol agents applied soybean plants might have conferred resistance against *M. phaseolina* by making physical barriers stronger or chemically impervious to the hydrolytic enzymes produced by the pathogen.
Fig. 1: Chitinase activity, peroxidase activity β-1, 3-glucanase, phenylalanine ammonia-lyase (PAL) activity and Phenolic content in leaves of soybean seedlings growing soil inoculated with plant growth promoting rhizobacteria and infected with *M. phaseolina*. NFB=Nitrogen fixing bacteria, BM= *Bacillus megaterium*, BC= *B. cereus*, PF= *Pseudomonas fluorescens*. 
Discussion:

Many researchers have used bacterial biological control as a means of protection against soil-borne plant diseases as an alternative control method to fungicides (Zavaleta, 2000, El-Barougy et al., 2009). In the presence study, all pots treated with NFB and B. cereus (BC) or Bacillus megaterium (BM) or Pseudomonas fluorescens (PF) and mixed bacteria decreased significantly damping-off as well as infested survival plants caused by M. phaseolina and increased healthy survival plants. Gupta et al (2002) found that Bacterization of peanut seeds with fluorescent Pseudomonas GRC2 resulted in increased seed germination, early seedling growth, fresh nodule weight, grain yield and reduced charcoal rot disease of peanut in M. phaseolina- infested soil as compared with control. A Landa et al., (2004) proved that seed and soil treatments with biocontrol agents Bacillus megaterium, B. subtilis and Pseudomonas fluorescens significantly reduced chickpea Fusarium wilt disease intensity and increased chickpea seed yield. Akhtar and Siddiqui (2008) they mentioned that Pseudomonas alcaligenes and Bacillus pumilus decreased disease incidence caused by M. phaseolina in chickpea plants. The mechanism by which bacterial biocontrol agents affecting fungal growth may be attributed to the presence of some effective substance such as antibiotics which play an important role in the biological control of plant diseases. Many investigators confirmed these results such as Liu and Sinclair (1989,1990) and Handelsman et al., (1990). Effect of bacterial plant growth promoting on root rot and wilt disease complex of soybean plant under field condition was also studied. Data showed that soil treated with NFB plus Bacillus cereus (BC) or Bacillus megaterium (BM) or Pseudomonas fluorescens (PF) significantly reduced diseased plants comparing with the control. The reduction reached 66.6, 35.5, 87.5 and 58.3% respectively, when recorded three months after sowing. But reached 30.5% from combined treatments of NFB plus BC and reached 63.3% from NFB plus BM while, 57.3% from NFB plus PF and 41.6% from the combined of all treatments.

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance wherein plant defenses are preconditioned by prior infection or treatment that results in resistance against subsequent challenge by a pathogen or parasite (Choudhary et al., 2007). The widely recognized mechanism of biocontrol mediated by PGPR is competition for an ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Haas et al., 2002).

Chitinase and peroxidase activities are commonly expressed during plant-fungus interactions (Dalisay and Kuc 1995, Heath 1996). On the other hand, peroxidase catalyses the condensation of phenolic compounds into lignin and is associated with disease resistance in plants and increases in host plants following pathogen infection (Scott-Craig et al., 1995). PGPR treatment initiated a marked increase in peroxidase activity within 6 days after inoculation. As a general rule, peroxidase activity increases earlier than chitinase activity in PGPR-treated soybean plants. Peroxidase may be rapidly involved in the peroxidation of substrate molecules, leading to the accumulation of highly toxic compounds (i.e., phenolic compounds), which may contribute to resistance via their antifungal potential (Ward 1986). However, these compounds may, to some extent, be toxic to the plant itself, and it seems reasonable to assume that mechanisms designed to repress peroxidase expression are activated during the resistance process in order to maintain phenolic compounds below phytotoxic levels. In that context, the decrease in peroxidase activity observed at 9 days post inoculation may reflect a process elaborated by the plant to protect itself until such activity is needed, such as upon pathogenic attack. Similar findings of increase in peroxidase proteins after application with biocontrol agents have been reported by several workers in different crops (Yedidia et al., 1999; Meena et al., 2000; Oostendorp et al., 2001). Yedidia et al., (1999) reported that inoculation of T. harzianum induced chitinase activities in both leaves and roots of cucumber seedlings. These proteins facilitate Trichoderma penetration into the host and the utilization of the host components for nutrition. The implication of lytic enzymes in biocontrol has been confirmed in overproducing mutants (Mendoza-Mendoza et al., 2003), and the expression of some of these enzymes in transgenic plants highly increased their resistance to different pathogens (Emani et al., 2003).

The increased accumulation of chitinase and β-1,3-glucanase due to the application of biocontrol agents, in addition to hydrolysing chitin and β-1,3 glucan respectively which are the major components of the fungal cell walls, might have also released elicitors from the walls of fungi which in turn might have triggered various defense related activities in soybean (Ren and West 1992). Yedidia et al. (1999) also observed increased accumulation of peroxidase due to root inoculation with T. harzianum in leaves of cucumber seedlings. Bacterization of pigeon pea seeds with B. subtilis increased the peroxidase activity from 1 to 7 day and reduced the Fusarium wilt incidence caused by F. udum (Podile and Laxmi, 1998). Morpurgo et al. (1994) also reported that the activity of peroxidase was at least five times higher in the roots and corn tissues of Foc resistant banana variety than in the susceptible variety. Inoculation of resistant plants with Macrophomina phaseolina resulted in a 10 fold increase in peroxidase activity after seven days of inoculation, whereas, the susceptible variety exhibited only a slight increase in peroxidase activity. The authors concluded that peroxidase activity could be
used as a parameter to discriminate between susceptible and tolerant clones of banana (Morpurgo et al., 1994). Phenylalanine ammonia-lyase (PAL) enzyme is associated with the production of specific phenolic compounds including some antifungal isoflavonoid phytoalexins and lignins (Vidhyasekaran, 1997). Plants treated with Pseudomonas strains 69-28 and 13 initially had higher levels of PAL, but when these plants were inoculated with the pathogens, the levels were reduced compared with other treatments or control (Chen et al., 2000). The authors concluded that early induction of PAL by PGPR might have resulted in the activation of defenses, but subsequent pathogen challenge did not induce higher PAL levels (Chen et al., 2000). Zdor and Anderson (1992) reported that when the endophytic bacterium B. pumilus strain SE34 was bacterized in roots of pea, it stimulated the strengthening of epidermal and cortical cell walls and deposition of newly formed barriers containing callose and phenolic compounds beyond the infection sites of F. oxysporum f. sp. pisi.

It can be concluded that colonization by PGPR confers a significant reduction in disease development. Soil application of PGPR increased the activities of defense enzymes and accumulation of phenols in soybean plants. In addition, challenge inoculation with M. phaseolina, also resulted in further increase of these enzymes and phenols in soybean leaves. This study indicated the usefulness of PGPR in protecting soybean plants against M. phaseolina by induction of systemic resistance.

References


