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Research Article

# *Pantoea agglomerans* ENA1 as a biocontrol agent of *Macrophomia phaseolina* and growth enhancer of soybean

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**Abstract:** On the basis of preliminary *in vitro* screening tests, a competent strain of *Pantoea agglomerans* ENA1 (P. agg. ENA1) recovered from soybean nodule was evaluated for its antagonistic activity against *Macrophomina phaseolina* causal agent of charcoal rot of soybean. The results of various *in vitro* assays showed that P. agg. ENA1 is capable of exerting strong antagonistic effect against *M. phaseolina* inhibiting its mycelial growth up to 89% as compared to control. The results showed a significant reduction of the disease as measured in host-plant weight increase, reduced microsclerotial coverage of the host tissues and decreased population of the pathogen in soil. Soils treated with the antagonist in presence of the pathogen resulted in 40% increase in aerial fresh weight and 63% decrease in root and stem surface covered by microsclerotia as compared with control. Furthermore, a significant decrease in the pathogen population ranging from 73 to 76% was observed in sterile and non-sterile soils, respectively. P. agg. ENA1 is suggested as a potent biocontrol agent that provides excellent rhizosphere colonization and control of *M. phaseolina*

**Keywords**: *Macrophomina phaseolina,* biocontrol, soybean, *Pantoea agglomerans,* antifungal metabolite

## Introduction

*Macrophomina phaseolina* (Tassi) Goid is a soil-and seed-borne polyphagous pathogen with an exceptionally broad host range. It causes charcoal rot and various rots and blight of more than 500 crop species of monocots and dicots (Dhingra and Sinclair, 1977; Sinclair and Backman, 1989). This pathogen is a serious problem of soybean, *Glycine max* (L.) Merr, in Golestan province of Iran.

*M. phaseolina* causes damage by plugging or rotting of vascular issue in roots and lower stems or stalks (Frederiksen, 1986; Sinclair and Backman, 1989) and heavily infected plants die prematurely due to the production of fungal toxins e.g. phaseolinone (Bhattacharya *et al*., 1994; Ndiaye, 2007). Microsclerotia in soil, host roots and stems are the main surviving propagules. They can survive for 2-15 years depending on environmental conditions (Cook *et al*., 1973; Papavizas, 1977; Dhingra and Sinclair, 1978; Baird *et al*., 2003). There are few strategies for control of charcoal rot in soybean. The main aim of the described control methods is to reduce the number of inoculums in soil or to minimize the contact of inoculums with hosts (Ndiaye, 2007). Few resistant genotypes have been found, however the rates of pathogen colonization maybe different among soybean cultivars (Pearson *et al*., 1984; Smith and Carvil, 1997). Crop rotation is not effective as a control tactic for charcoal rot because this fungus has a wide host range (Mihail, 1992). Irrigation at any time during the cropping season reduces disease

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incidence in soybean (Kendig *et al*., 2000) and one summer irrigation was sufficient to reduce the population of *M. phaseolina* by 25-42% (Lodha and Solanki, 1992; Lodha, 1995). In general, recommended chemicals and seed conservation are not efficient in controlling charcoal rot disease under field conditions because the crop is vulnerable to pathogen attack at any growth stage (Pearson *et al*., 1984; Singh and Kaiser, 1995). Thus, several studies have considered using of biocontrol agents against of *M. phaseolina*. PGPR promote plant growth directly or indirectly via biological control of pathogens, production of phytohormones and antagonistic activity by antibiosis, hyperparasitism and competition for nutrients and space (Chet *et al*., 1990; Whipps, 1992; Handelsman and Stabb, 1996; Shoda, 2000). Several strains have suppressed *M. phaseolina* in other hosts under *in vitro* or field conditions. These include *Bacillus subtilis* (Siddique and Mahmood, 1993), *Bacillus* spp. (Omar *et al*., 2013), *Rhizobium meliloti* (Arora *et al*., 2001), *Bradyrhizobium* sp. (Deshwal *et al*., 2003) and *Paenibacillus* sp. HKA-15 (Senthilkumar *et al*., 2007).

In present work, we studied the plant growth promoting and antifungal activities of *Pantoea agglomerans* (syn: *Erwinia herbicola, Enterobacter agglomerans*) on soybean charcoal rot. Unfortunately, little attention has been given to the potential value of these bacteria for control of soil-borne plant disease fungal agents. Some such reports include antagonistic effects of *P. agglomerans* against *Fusarium culmorum* and *Puccinia recondita* f.sp. *tritici* (Kempf and Wolf, 1989), *Rhizoctonia solani* (Chernin *et al*., 1995), *Botrytis cinerea* and *Penicillium expansum* (Bryk *et al*., 1998; Nunes *et al*., 2001; Morales *et al.,* 2008), *Fusarium moniliforme* (Hebbar *et al*., 1992a), *Penicillium digitatum* (Plaza *et al*., 2004), *Monilinia laxa* (Franc′es *et al*., 2006) and *Aspergillus flavus* (Kotan *et al*., 2009).

## Materials and Methods

### Isolation and identification of *M. phaseolina*

During 2006, 11 isolates of *M. phaseolina* (M21, M16, M13, MK1, ML1, MA1, MS1, MN1, MB1, MT1 and MG1) were isolated from diseased soybean plants of eight regions of Golestan province, Iran (Aghghala, Lemesk, Sarkalateh, Toskestan, Kafshgiry, Nasrabad, Khanbebin and Kordkoy). Isolates were maintained on Potato Dextrose Agar (PDA). All cultures were incubated at 28 °C in darkness and identified, based on morphological characters, as *M. phaseolina* and confirmed by species specific primers MpKF1 (5′-CTCAAACAGGCATGCTC-3′) and MpKR1 (5′-AGCAATAGTTGGTGGA-3′) (Babu *et al*., 2007). The pathogenicity of *M. phaseolina* isolates was determined on William′s soybean cultivar in greenhouse (Vasebi, 2008). Isolate of *M. phaseolina* M21 was determined as the most virulent.

### Isolation of bacteria

Two healthy soybean plants were collected from Aghghala fields in Golestan province, Iran in 2006,t heir root nodules were detached,sterilized with 2% NaOCl for 20 secand, rinsed in sterile distilled water (4 times, 3 min). Nodules were crashed and streaked on Nutrient Agar Medium (NA). The dishes were incubated at 26 °C for 48 h. All bacterial colonies had the same morphology. Five colonies were selected and introduced as ENA1, ENA2, ENA3, ENA4 and ENA5. These colonies were purified and maintained on NA at 4 °C. The isolates were characterized following morphological, physiological and biochemical parameters (Schaad *et al*., 2001).

### Selection of antagonists

Antagonistic activity of bacterial strain was tested against *M. phaseolina* by using dual culture technique. Each bacterial suspension ($10^9$ cfu/ml) was cultured in a circular pattern on the inner periphery of the Petri dishes (9 cm) containing fresh PDA. After 24 and 72 hours of bacterial growth, a plug of 3-day-old PDA culture of *M. phaseolina* with mycelium and microsclerotia was placed at the center of each Petri dish. Distilled water was smeared in the circular pattern in the control dishes. The dishes were incubated at 28 °C until mycelial growth of *M. phaseolina* reached on the inner periphery of the control Petri dishes. The percent of *M. phaseolina* inhibition growth by bacterial

strains was calculated using the formula (1) during three days.

(1) $IG = ((C-T) / C) \times 100$, where IG was percentage of growth inhibition, and C and T were radial growth in control and treatment respectively.

**Antibiotic production**

Production of antibiotic was determined by Kraus and Lopper (1990) method. The Petri dishes were incubated at 28 °C for three days. The examination was done with three replications in completely randomized design. The percent inhibition of mycelial growth was calculated by formula (1).

**Volatile production**

Production of volatile metabolites was estimated by the method of Fernando *et al*. (2005). Petri dishes were incubated at 28 °C for three days. The *M. phaseolina* growth inhibition was compared with control using mentioned formula after three days.

**Extra cellular metabolite production**

Another set of inhibition assay was performed with cell-free culture filtrate (CFCF) of the bacteria (Singh and Deverella, 1984). Log phase culture of bacterial strains was produced in TSB medium (Triptych Soy Broth) incubated for 24 h. Spent medium was collected by centrifugation at 6000 g for 20 min at 4 °C. The supernatant was collected and passed through 0.22 µm Millipore filter. 15 ml of sterile PDA 45-50 °C was mixed with 5ml of cell-free culture filtrate antagonist. One 3day-old mycelium disc (5 mm dia) of *M. phaseolina* was placed at the center of dishes and incubated at 28 °C for three days. A similar experiment was done for non-antagonistic bacteria CFCF as control. After three days the growth of the pathogen exposed to extracellular metabolites was compared with that of control and the growth inhibition was calculated.

**Siderophore production**

Siderophore production was estimated by the modified method of Alexander and Zubrer (1991) using CAS-agar medium (Chrome Azural Agar). A loop of bacterial suspension ($10^9$ cfu/ml) was placed at the center of Petri dishes. The dishes were incubated at 26 °C for four days. Then the production of orange halo around the bacterial colonies was evaluated.

**IAA production**

Two drops of *o*-phosphoric acid were added to 2 ml of cell-free culture filtrate of antagonist isolate. An antagonist isolate with ability of IAA production was used as positive control. Appearance of pink color was indicative of IAA production (Gupta *et al*., 2002).

**Preparation of mutant isolate**

To determine the population dynamics of antagonist during greenhouse experiments, antibiotic-resistant mutant was prepared. A Rifampicin and Nalidixic acid-resistant (200 µg $ml^{-1}$) strain was selected by passing the antagonist isolate through the lowest to highest concentrations (5-10-20-50-100-135-150-175 and 200 µg$ml^{-1}$) of Rifampicin and Nalidixic acid on NA medium. Resistance of mutant to antibiotics was confirmed by culturing in NB without antibiotics (10 times) and transferring it to the NA medium containing antibiotics.

**Selection of fungicide**

Maneb (wp 80%), thiabendazol (wp 60%) and captan (wp 75%) fungicides were used against *M. phaseolina in vitro*. Four concentrations of these fungicides (0.5, 1, 1.5 and 2 g $l^{-1}$) were prepared in PDA medium and a 3-day-old plug of pathogen culture was placed in the center of Petri dishes. PDA without fungicide served as control for each fungicide. The Petri dishes were incubated at 28 °C for five days and evaluated for growth of *M. phaseolina*.

**Evaluation of antibiotic encoding genes in *P. agglomerans***

For detecting pyrrolnitrin-encoding genes in wild type and mutant isolates of antagonist, specific primers PrnAR (5'-TGCCGGTTCGCGAGC CAGA-3') and PrnAF (5'–GTGTTCTTCGAC

TTCCT-3') were used in polymerase chain reaction (PCR) (Zhang, 2004).

**Greenhouse tests**

The plastic pots (17 × 20 × 20) were filled with sterile or non-sterile sandy soil, prelate and peat moss (1:1:1). Inoculums of *M. phaseolina* were prepared by growing the pathogen on rice grains. The grains were soaked in distilled water, autoclaved twice (121 °C for 45 min) and inoculated with three agar discs (5 mm dia) of 5-day-old pathogen culture. Flasks (250 ml) were incubated at 28 ± 1 °C in dark for 15 days. Then the inoculums were mixed with soil (10 g $kg^{-1}$ soil) completely. Four soybean seedlings with three leaves grown on peat moss were transplanted in each pot. The potted plants were kept at 25-33 °C and allowed to grow up to 100 days. Bacterial strains were applied in soil as suspension. Antagonists were grown in 250 ml NB (Nutrient Broth) at 26 °C for 48 h with shaking at 150 rpm. The cells were harvested and adjusted to $10^9$ cfu $ml^{-1}$ (125 ml, $10^9$ cfu $ml^{-1}$). 125ml of both strain and fungicide were added to pots every 14 days after planting.

There were 16 treatments in each experiment with three replications, which included: a) control; b) pathogen; c) wild type antagonist; d) mutant antagonist; e) pathogen with wild type antagonist; f) pathogen with mutant antagonist; g) fungicide; h) pathogen with fungicide in sterile and non-sterile soils. Variables such as: root and aerial fresh and dry weight s(gr); and percentage of microsclerotial coverage on roots and stems were estimated. The experiment was conducted twice during 2007-2008 in randomized complete block design. Data were analyzed by MSTATC to evaluate the efficiency of biocontrol treatments.

**Monitoring of introduced antagonist and pathogen**

Evaluation of population dynamics of biocontrol agent and pathogen was done via sampling of soybean rhizosphere soil containing root hairs every seven days after application of antagonist suspension and fungicide. Antagonist population was counted using serial dilution method on NA medium containing Rifampicin and Nalidixic Acid. Nutrient agar medium without antibiotics was used as control. The plates were kept at 26 °C for 72 h. For monitoring of the pathogen, Rose Bengal medium containing 200 ppm Chloramphenicol was prepared. Petri dishes were incubated at 28-30 °C for 48 h. The population of antagonist and pathogen (cfu/gr soil) were then counted

**Results**

**Characterization of bacteria strain**

All of the 5 selected isolates, ENA1 to 5, were identified as *Pantoea agglomerans* (= *Erwinia herbicola*) based on standard tests according to Schaad *et al*. 2001 (Table 1). The ENA1 isolate was selected for *in vitro* and *in vivo* experiments.

***In vitro* experiments**

In dual culture test ENA1 reduced growth of the pathogen more than 43 and 62% in 24 and 72 hours tests, respectively. In antibiotic production test on solid media, ENA1 inhibited the mycelial growth of *M. phaseolina* more than 89%. *P. agglimerans* ENA1cell-free culture filtrate reduced the pathogen growth 12%. Volatile metabolites were produced by ENA1 and inhibited the growth of *M. phaseolina* more than 34.5%. Siderophore production by the antagonistic strain was detected by observing orange zone around the bacterial colonies on CAS-agar medium. The 24-hour-old culture of ENA1 showed an orange halo with 23.8 mm diameters after 4 days (Table 2). Results showed that *P. agglomerans* ENA1 wasn't able to produce IAA in presence of α-phosphoric acid in compared to control.

Evaluation and detection of pyrrolnitrin antibiotic encoding genes in wild type and mutant (Rifampicin and Nalidixic acid-resistant) strains of *P. agglomerans* ENA1 showed that both of them had the desired genes and that a fragment of 1050 bp was amplified in wild type and mutant isolate by PrnAR/PrnAF specific primers (Fig. 1).

**Table 1** Morphological, physiological and biochemical characteristics of *Pantoea agglomerans* ENA1, ENA2, ENA3, ENA4 and ENA5 isolated from soybean nodules.

| Characteristics | ENA | Characteristics | ENA |
|---|---|---|---|
| Gram reaction | - | Nitrate reduction | + |
| Anaerobic growth | + | Gelatin liquefaction | + |
| Fluorescent pigment on KB | - | Motility | + |
| Spore formed | - | Urease | - |
| Aerial mycelium | - | Oxidase | - |
| Tobacco hypersensitivity | - | Utilization of Citrate | + |
| Yellow pigment | + | Acid production from: | |
| Yellow pigment on YDC | - | Arabinose | + |
| Taupe pigment on YDC | - | Lactose | + |
| Growth at 37 °C | + | Maltose | + |
| $H_2S$ from cysteine | + | Raffinose | + |
| Indole production | - | Sorbitol | - |

+: positive reaction; -: negative reaction.

*In vitro* selection of an effective fungicide against *M. phaseolina* and for control of soybean charcoal rot showed that maneb in all applied concentrations completely inhibited the pathogen mycelial growth (100%) but thiabendazol and captan didn't reduce mycelia growth of the pathogen in any of the cocentrations tested. Thus 1 g $l^{-1}$ concentration of maneb was used in greenhouse experiments.

### *In vivo* studies

Use of wild type strain in the pots inoculated with *M. phaseolina* resulted in increasing 40% of soybean aerial fresh weight in sterile soil compared with control, 100 days after planting (Fig. 2). The effects of wild type and mutant strains on root and stem microsclerotial coverage in sterile and non-sterile soils were similar to that of maneb fungicide treatment. The wild type strain in sterile and non-sterile soils decreased microsclerotial coverage of *M. phaseolina* 62.5 and 73%, respectively. The mutant strain decreased root and stem microsclerotial coverage 50 and 82% in sterile and non-sterile soils, respectively.

High percent reduction in microsclerotial coverage on soybean root and stem in non-sterile soil in presence of *P. agglomerans* ENA1 showed that the other soil microorganisms had positive antagonistic effects in combination with *P. agglomerans* ENA1 against *M. phaseolina*. On the other hand, maneb decreased the microsclerotial coverage of *M. phaseolina* in sterile (87.5%) and non-sterile (73%) soils compared to control (Table 3). In the presence of antagonist, the population of *M. phaseolina* within 49 days decreased 73% and 76% in sterile and non-sterile soils, respectively. Maneb reduced the population of pathogen by 57 and 60% in sterile and non-sterile soils, respectively. The statistical analysis showed non-significant difference between antagonist and maneb effects on population dynamics of *M. phaseolina* (Tables 4 and 5; Fig. 3). Bacterial effects on plant growth factors were similar to their effects on population dynamic and microsclerotial formation of pathogen in rhizosphere and on soybean roots.

**Table 2** Inhibition of mycelial growth of *Macrophomina phaseolina in vitro* assays by *Pantoea agglomerans* ENA1 and production of siderophore.

| Entries | Dual culture (%) | | Antibiotic production (%) | Volatile metabolite (%) | Extra-cellular metabolite (%) | Siderophore production (mm) |
|---|---|---|---|---|---|---|
| | 24h | 72h | | | | |
| ENA1 | 43* | 62** | 89** | 34.5** | 12** | 23.8 |
| Control | 0 | | 0 | 0 | 0 | 0 |

Data are the means of three replicates. **: $p < 0.01$

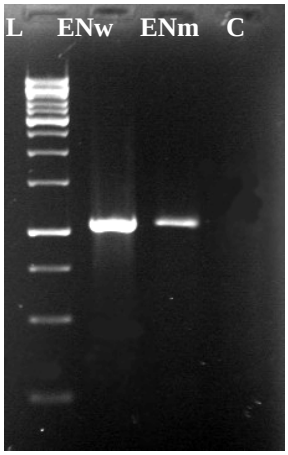


**Figure 1** Agarose gel electerophoresis of PCR-amplified gene coding pyrrolnitrin antibiotic in wild type of *Pantoea agglomerans* ENA1 and its derivative mutant. L: 1 kb DNA lader; ENw: wild type isolate; ENm: mutant isolate; C: non-antagonist bacteria isolate (control).

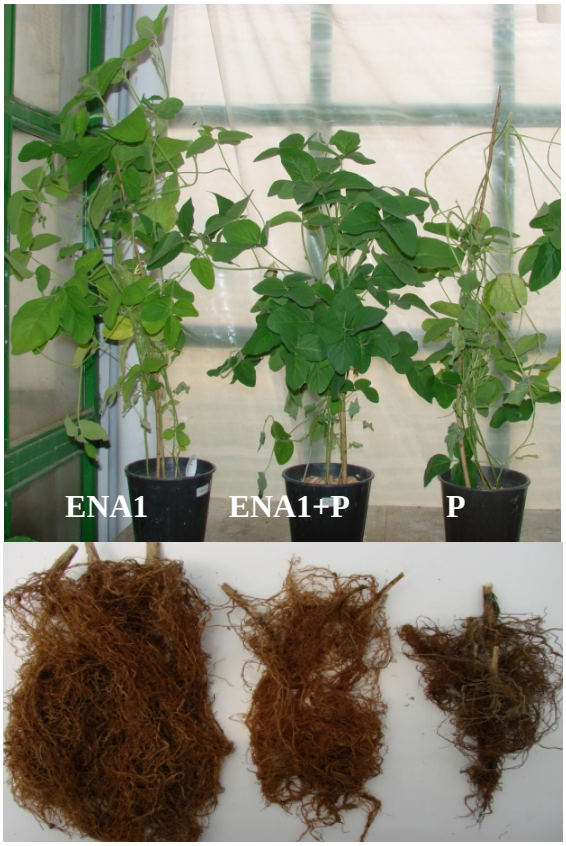


**Figure 2** Effects of *Pantoea agglomerans* ENA1 on aerial parts and roots of soybean alone and in presence of *Macrophomina phaseolina* in greenhouse experiment. ENA1: *P. agglomerans* ENA1, P: *M. phaseolina.*

**Table 3** Effects of wild type and mutant strains of *Pantoea agglomerans* ENA1 and maneb fungicide alone and in combination with *Macrophomina phaseolina* on soybean growth factors in sterile and non-sterile soils in greenhouse assays after 100 days.

| Treatment | FRW (g / pot) | FAW (g / pot) | DRW (g / pot) | DAW (g / pot) | MC (%) |
|---|---|---|---|---|---|
| **C/S** | 38.50 a | 168 ab | 6.3 ab | 60 a | - |
| **C/NS** | 27.15 bc | 131 bc | 5.7 bc | 44 ab | - |
| **P/S** | 15.92 defg | 112 c | 3.2 g | 37 b | 53.3 ab |
| **P/NS** | 19.43 cdefg | 132 bc | 4.3 defg | 42 ab | 73.3 a |
| **Nw/S** | 22.95 cdef | 181 a | 5.3 bcd | 49 ab | - |
| **Nw/NS** | 24.47 bcde | 156 ab | 5.4 bcd | 43 ab | - |
| **Nw/P/S** | 12.43 g | 156 ab | 3.3 fg | 51 ab | 20.0 bc |
| **Nw/P/NS** | 15.20 efg | 136 bc | 3.6 fg | 39 b | 20.0 bc |
| **Nm/S** | 21.00 cdefg | 157 ab | 4.4 def | 49 ab | - |
| **Nm/NS** | 21.12 cdefg | 167 ab | 4.3 defg | 52 ab | - |
| **Nm/P/S** | 14.00 fg | 142 abc | 4.0 efg | 42 ab | 26.6 bc |
| **Nm/P/NS** | 13.83 fg | 146 abc | 3.7 fg | 36 b | 13.3 bc |
| **F/S** | 27.43 bc | 144 abc | 5.9 abc | 43 ab | - |
| **F/NS** | 33.20 ab | 140 bc | 7.0 a | 41 b | - |
| **F/P/S** | 26.28 bc | 128 bc | 5.9 abc | 38 b | 6.6 c |
| **F/P/NS** | 25.27 bcd | 134 bc | 5.0 cde | 38 b | 20.0 bc |

Different letters in the same column indicate significant differences between means using Fisher's LSD test ($p < 0.05$). Data are the means of three replications. Each replication is included of four seedlings in a pot. FRW: Fresh Root Weight; DRW: Dry Root Weight; FAW: Fresh Aerial part Weight; DAW: Dry Aerial part Weight; MC: Microsclerotial Coverage; Nw: wild type strain; Nm: mutant strain; P: pathogen; S: sterile soil; NS: non sterile soil; C: control.

**Table 4** Population of *Pantoea agglomerans* ENA1 in soybean rhizosphere compared to detectable total bacterial population alone and in combination with *Macrophomina phaseolina* in sterile and non sterile soil during 49 days.

| Treatment | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| | A | T | A | T | A | T | A | T |
| | Population of bacteria in rhizosphere of soybean in sterile soil (CFU $g^{-1}$ soil) | | | | | | | |
| *P. agglomerans* | $7.2 \times 10^{7*}$ | $9.4 \times 10^{7}$ | $1 \times 10^{7**}$ | $10.2 \times 10^{7}$ | $0.35 \times 10^{7*}$ | $13 \times 10^{7}$ | $0.86 \times 10^{7**}$ | $7.9 \times 10^{7}$ |
| *P. agglomerans* + *M. phaseolina* | $1.7 \times 10^{7**}$ | $18.2 \times 10^{7}$ | $0.5 \times 10^{7**}$ | $11.7 \times 10^{7}$ | $0.41 \times 10^{7**}$ | $16 \times 10^{7}$ | $0.45 \times 10^{7**}$ | $8.4 \times 10^{7}$ |
| | Population of bacteria in rhizosphere of soybean in non sterile soil (CFU $g^{-1}$ soil) | | | | | | | |
| *P. agglomerans* | $0.7 \times 10^{7*}$ | $8.6 \times 10^{7}$ | $0.46 \times 10^{7**}$ | $8.7 \times 10^{7}$ | $0.37 \times 10^{7**}$ | $7.7 \times 10^{7}$ | $0.54 \times 10^{7**}$ | $5.9 \times 10^{7}$ |
| *P. agglomerans* + *M. phaseolina* | $2.5 \times 10^{7**}$ | $12.1 \times 10^{7}$ | $0.86 \times 10^{7**}$ | $9.8 \times 10^{7}$ | $0.23 \times 10^{7**}$ | $7.37 \times 10^{7}$ | $0.30 \times 10^{7**}$ | $5.0 \times 10^{7}$ |

Data are the means of three replicates. *CFU*: Colony-forming unit. A: antagonist (*P.agglomerans* ENA1); T: total bacteria; 1, 2, 3, 4: Detachment periods (every 14 days). $^{*}$: $p < 0.05$; $^{**}$: $p < 0.01$.

**Table 5** Population dynamics of *Macrophomina phaseolina* in soybean rhizosphere alone and in presence of antagonist (*Pantoea agglomerans* ENA1) and fungicide (maneb) in sterile and non sterile soils (CFU $g^{-1}$ soil).

| Treatment | Population of *Macrophomina phaseolina* (CFU $g^{-1}$ soil) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | |
| | S | NS | S$^{*}$ | NS | S | NS | S | NS |
| *M. phaseolina* | $13.3 \times 10^{3ns}$ | $6.3 \times 10^{3ns}$ | $26.6 \times 10^{3**}$ | $8.6 \times 10^{3**}$ | $8.3 \times 10^{3**}$ | $5.0 \times 10^{3**}$ | $12.0 \times 10^{3**}$ | $6.3 \times 10^{3**}$ |
| *M. phaseolina* + *P. agglomerans* | $15.0 \times 10^{3ns}$ | $13.5 \times 10^{3ns}$ | $6.6 \times 10^{3**}$ | $5.0 \times 10^{3**}$ | $3.5 \times 10^{3**}$ | $2.0 \times 10^{3**}$ | $4.0 \times 10^{3**}$ | $3.5 \times 10^{3**}$ |
| *M. phaseolina* + Maneb | $5.3 \times 10^{3ns}$ | $5.0 \times 10^{3ns}$ | $7.3 \times 10^{3**}$ | $4.8 \times 10^{3**}$ | $3.5 \times 10^{3**}$ | $1.6 \times 10^{3**}$ | $2.3 \times 10^{3**}$ | $2.0 \times 10^{3**}$ |

Data are the means of three replicates. *CFU*: Colony-forming unit. S: sterile soil; NS: non sterile soil; 1, 2, 3, 4: Detachment periods (every 14 days). $^{**}$: $p < 0.01$, $^{ns}$: non-significant difference.

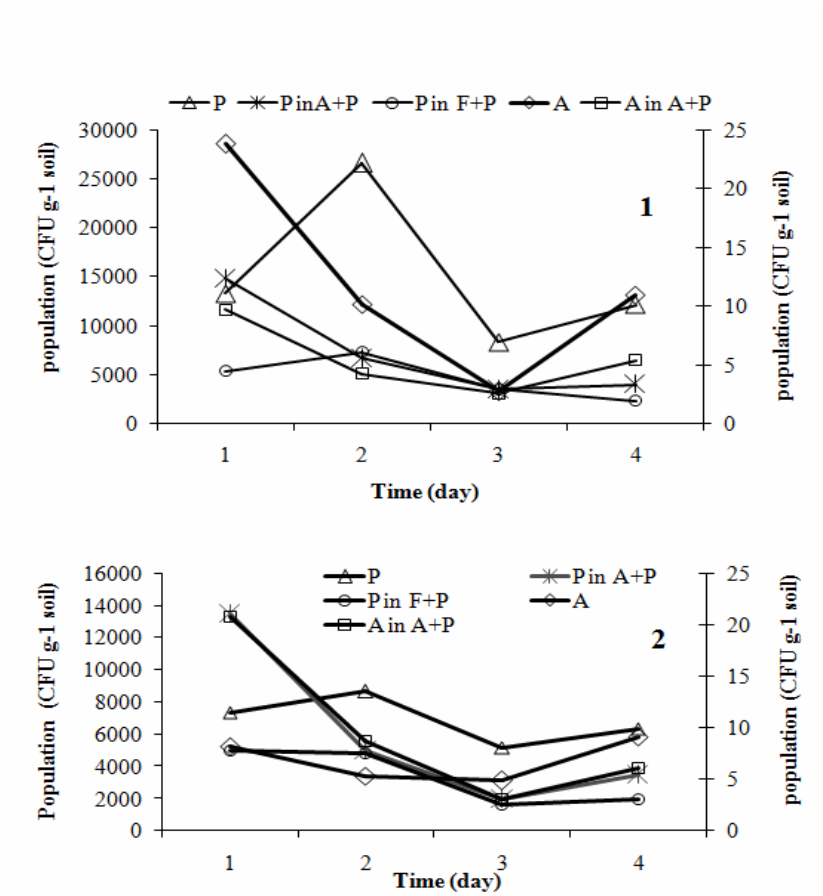


**Figure 3** Population dynamics of *Pantoea agglomerans* ENA1 alone (A) and in presence of *Macrophomina phaseolina* (A in A + P), *M. phaseolina* alone (P) and in presence of *P. aglomerance* ENA1 (P in A + P), maneb fungicide in presence of *Macrophomina phaseolina* (P in F+P) in rhizosphere of soybean in sterile soil (1) and in non-sterile soil (2) within 49 days. A: *P. agglomerans* ENA1; P: *M. phaseolina*; F: maneb.

## Discussion

Despite several reports on suppression of *M. phaseolina* charcoal rot by different rhizobacteria like *Pseudomonas fluorescens* (Gupta *et al*., 2002), *Bacillus subtilis* BN1 (Singh *et al*., 2008) and *Rhizobium meliloti* (Anis *et al*., 2010), any studies have not been performed on the biological control of this pathogen with *P. agglomerans*. *P. agglomerans* is a common epiphytic bacteria (Cook and Baker, 1983) that has been reported as a biocontrol agent against plant pathogens (Montesinos *et al*., 1996; Zhang and Birch, 1997; Stockwell *et al*., 1998) and postharvest diseases of fruits (Bonaterra *et al*., 2003; Trotel-Aziz *et al.,* 2008).

A large number of soil microorganisms are capable of producing siderophores. Moreover siderophores may not be produced in sufficient quantities in the soil microcosms to have any significant biocontrol effect (Misaghi *et al*., 1988), while antibiotics, antifungal volatiles and other metabolites are involved in suppression of *M. phaseolina* (Hebbar *et al*., 1992b; Gupta *et al*., 2002). The high ability of *P. agglomerans* ENA1 in siderophore production in CAS-agar medium has been confirming that this group of bacteria has evolved high-affinity iron uptake systems to shuttle iron into the cell. It has been shown earlier that some enterobacter genera, i.e. *Erwinia*, *Pantoea*, *Enterobacter*,

*Hafnia* and *Ewingella* also synthesize ferrioxamines E, D and G under iron limitation (Berner *et al*., 1988; Reissbrodt *et al*., 1990). These reports indicate a great number of naturally occurring enterobacter genera are equipped with ferrioxamine biosynthesis and uptake systems (Deiss *et al.,* 1998). *Pantoea* sp. strain 48b/90 isolated from soybean leaf produced two different siderophores (the known ferrioxamine E and a non-identified catechol siderophore) and a stabile antibiotic in chemically defined medium (Völksch and Sammer, 2008).

The primary biocontrol mechanism by PGPR involves the production of antibiotics. Several rhizobacteria and bacterial epiphytes of plants, such as *Pseudomonas flourescens* and *P. agglomerans* produce multiple antibiotics against plant pathogenic fungi and bacteria, and have been used as biocontrol agents of some disease in the phyllosphere and the rhizosphere (Montesinos *et al*., 1996). Reducing of *M. phaseolina* mycelial growth 89% by antibiotic production could be considered that antibiosis was one of the main mechanisms of this biocontrol agent. Application of specific primers PrnAF/PrnAR in wild type and mutant strains of *P. agglomerans* ENA1 indicated that both strains carried pyrrolnitrin encoding gene. Chernin *et al*. (1996) showed that the purified pyrrolnitrin antibiotic produced by *Enterobacter agglomerans* IC1270 (*Serratia plymuthica*) was efficient against many phytopathogenic bacteria and fungi *in vitro*. Pyrrolnitrin (PRN) is a chlorinated phenylpyrrole antibiotic produced by several fluorescent and non-fluorescent Pseudomonads.

The mechanism by which *P. agglomerans* reduces decay is not clear. It has been postulated *P. agglomerans* inhibits plant pathogens by colonization of them and competition for nutrients (Kempf and Wolf, 1989; Amellal *et al*., 1998), parasitism (Bryk *et al.,* 1998) and production of antibiotics and siderophore (Kearns and Mahanty, 1998; Stockwell *et al*., 2002).

In evaluation of antagonistic effect on soybean growth factors in presence and absence of pathogen, in sterile and non-sterile soils, there was some statistically significant difference between wild type and mutant strain treatments. The wild type strain in sterile soil was most effective on aerial fresh and dry weight of soybean with 40 and 39%increase respectively, compared to control. The mutant strain in presence of pathogen in sterile soils increased the aerial fresh and dry weight by 27 and 14.5 % respectively, compared to control. Evaluation of population dynamics of antagonist showed that *P. agglomerans* ENA1 was able to colonize the rhizosphere of soybean and increased its population well. During the experiment, increasing of ENA1strain population indicated that the strain was potential root colonizer that decreased the population dynamics of pathogen by colonizing soybean rhizosphere and suppressed the effects of *M. phaseolina* on soybean growth factors compared to control. Available literature reveals that the legume symbiotic bacteria enhance the host growth over other bacteria and show synergism with them, if they are able to reduce root disease (Deshwal *et al*., 2003). Therefore, it could be better if legumes are inoculated with host-specific rhizobia species, which provide not only nitrogen but also some degree of protection against seed-borne and soil-borne phytopathogens.

Fungicide application had a significant effect on plant growth improvement in comparison with control in sterile and non-sterile soils. Maneb reduced *M. phaseilina* population in soil and microsclerotia formation on root and stem of soybean. But application of benomyl and captan, as soil drench was ineffective against *M. phaseolina* (Valiente *et al*., 2008). Nunes *et al*. (2001) have stated that biocontrol agent *P. agglomerans* CPA-2 could be used as a substitute for chemicals such as imazalil to control *Penicillium expansum* and *Botrytis cinerea*.

PGPR must grow on, in or around the roots for the colonization of plant roots, which is of

primary importance for an effective plant-microbe interaction (Kleopper and Beauchamp, 1992). It was observed that rhizobacteria isolates could colonize successfully the rhizosphere at *In vivo* experiments. Evaluation of population dynamics of biocontrol agent using antibiotic markers showed the proper colonization of the *P. agglomerans* ENA1 in the rhizosphere.

Improved plant growth factors (i.e. root and aerial fresh and dry weight) and decreased percent of microsclerotial coverage on root and stem were observed 100 days after inoculation. In *M. phaseolina*-infested soils, the plants showed charcoal rot symptoms clearly when harvested after 100 days. Profuse mycelial growth and sclerotia were clearly visible beneath the epidermis of the root and collar region of infected plants. Narula *et al*. (2007) observed an overall increase in plant growth parameters under greenhouse conditions when they inoculated wheat with *Azotobacter chroococcum* and *Pantoea agglomerans* D5/23 strain. The endophytic strain of *Pantoea agglomerans* YS19 in rice plant promoted host growth and affected allocation of host phytosynthates (Feng *et al*., 2006). Study of antagonistic activity of PGPR against *M. phaseolina* on soybean in pot and field experiments indicated that all tested PGPR were significantly decreased damping-off, rotted and wilted plants and increased healthy plants compared to the control (El-Barougy *et al*., 2009). Root colonization is one of the most important steps in the interaction of bacteria and host plants. (Weller, 1988). The marketed strain *P. agglomerans* $^{rif\ +\ nal\ +}$ ENA1 showed excellent ability in colonization of soybean root and rhizosphere and this resulted in enhancing vegetative parameters and suppressing charcoal root rot disease of soybean and decline the *M. phaseolina* population (Gupta *et al*., 2002; Deshwal *et al*., 2003; Singh *et al.,* 2008). *In vitro* and *in vivo* attributes of *P. agglomerans* ENA1 verifies it as a potent biocontrol agent against *M. phaseolina*. Further researches will involve studies on the control mechanisms of *P. agglomerans* ENA1on charcoal rot agent, especially microsclerotia formation in field.

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# *Pantoea agglomerans* ENA1 عامل بیوکنترل *Macrophomina phaseolina* و محرک رشد سویا

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**چکیده:** براساس آزمون‌های غربالگری اولیه در آزمایشگاه، جدایه (P. agg. ENA1) *Pantoea agglomerans* ENA1 جدا شده از گره‌های ریشه سویا به‌عنوان جدایه آنتاگونیست بر علیه *Macrophomina phaseolina* عامل پوسیدگی ذغالی سویا انتخاب گردید. نتایج آزمونهای مختلف درون شیشه‌ای اثرات بالای آنتاگونیستی P. agg. ENA1 بر علیه *M. phaseolina* را نشان داد. به‌طوری‌که در مقایسه با شاهد رشد ریسه‌ای بیمارگر را تا ۸۹٪ کاهش داد. در آزمون‌های گلخانه‌ای افزایش معنی‌دار وزن گیاه میزبان، کاهش پوشش میکرواسکلروتی بافت‌های میزبان و کاهش جمعیت بیمارگر در خاک حاصل گردید. خاک‌های تیمار شده با آنتاگونیست در حضور بیمارگر افزایش ۴۰٪ وزن تر اندام‌های هوایی و کاهش ۶۳٪ پوشش میکرواسکلروتی ریشه و ساقه را در مقایسه با شاهد نشان دادند. همچنین جمعیت بیمارگر به‌طور معنی‌داری به میزان ۷۳٪ و ۷۶٪ به‌ترتیب در خاک‌های استریل و غیراستریل کاهش یافت. ایجاد پوشش مناسب در ریزوسفر و بازدارندگی بالا از رشد *M. phaseolina*، جدایه P. agg. ENA1 را به‌عنوان عامل بیوکنترل قوی معرفی می‌نماید.

**واژگان کلیدی**: *Macrophomina phaseolina*، بیوکنترل، سویا، *Pantoea agglomerans*، متابولیت‌های ضدقارچی