Rhizobium japonicum as a Biocontrol Agent of Soybean Root Rot Disease Caused by *Fusarium solani* and *Macrophomina phaseolina*

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Abstract

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The activity of *Rhizobium japonicum* against the soil-borne pathogens *Fusarium solani* and *Macrophomina phaseolina* as causative agents of soybean root rot disease in both culture medium and soil was evaluated. Rhizobial culture filtrate caused an inhibition of the fungal radial growth of *Fusarium solani* and *Macrophomina phaseolina* on potato dextrose agar medium amended with the filtrate compared with control. The addition of rhizobial culture suspension to the soil contaminated by the two pathogens, *Fusarium solani* and *Macrophomina phaseolina* and their interaction, in pots, improved seed germination percentages and reduced the root rot disease index significantly. The sowing of rhizobial coated seeds in soil contaminated by *Fusarium solani* and *Macrophomina phaseolina* separately and in combination, in the field, increased seed germination significantly and induced a high reduction in disease severity for the same previous combination under field conditions. These results indicate that rhizobia could be an important element in root rot disease management.

Keywords: biological control, *Rhizobium* sp.; soybean; *Fusarium*; *Macrophomina*

Soybean (*Glycine max* L. Merill) is one of the most important legume crops in the world representing a major component of the diet of food-producing animals and humans (Hapgood 1987; Friedman & Brandon 2001). Soybean oil was found to induce an increase in bird's weight when added to broiler diet (Scaife *et al*. 1994; Vieira *et al*. 2002; Lara *et al*. 2003).

It has been reported that soybean plants are subject to the infection by several soil-borne pathogens, inducing root rot disease, which is considered among the most important limiting factors affecting plant growth and yield (Cook *et al*. 1995; Yang & Feng 2001); *Fusarium solani* and *Macrophomina phaseolina* are among the most important of them (Zemanková & Lebeda 2001; Veverka *et al*. 2008). Due to great harms caused by chemicals used to control soil-borne pathogens, to environment and health, biological control using non-pathogenic microorganisms was adopted as an alternative to chemicals for combating these pathogens.

Saprophytic rhizosphere bacteria are present in large numbers on the plant root surface using root exudate and lysate as nutrients for growth. Certain of these bacteria can stimulate plant growth through releasing secondary metabolites which facilitate the uptake of certain nutrients from the root environment, so they are referred to as plant growth promoting rhizobacteria (PGPR) as well as suppressing soil-borne pathogens in the rhizosphere (Bakker *et al*. 2007).

The colonization of plant roots by plant growth promoting rhizobacteria and other factors and treatments can elicit the host defence against

various pathogens as indicated by a reduction of disease incidence and severity which constitute a state of induced systemic resistance in the plants to subsequent pathogen attack (Hammerschmidt 1999; Kloepper *et al*. 2004; van Loon 2004; Al-Ani *et al*. 2010, 2011a,b,c; Diwan *et al*. 2011). PGRR isolated from the rhizosphere soil have been shown to inhibit plant pathogens through competition for nutrients, competition for iron by siderophores, antibiosis or secretion of lytic enzymes as well as inducing systemic resistance (ISR) (Jogaiah 2010). Of these PGPR, rhizobia are reported as effective biocontrol agent for the inhibition of soil-borne plant pathogens. Many species of rhizobia were found to promote plant growth and also to inhibit the growth of various soil-borne pathogens including *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium* sp. in both leguminous and non-leguminous plants (Ehteshamul-Haque & Ghaffar 1993; Sharif *et al*. 2003; El-Batanony *et al*. 2007).

The present study was conducted to evaluate the antagonistic activity of *Rhizobium japonicum* against *Fusarium solani* and *Macrophomina phaseolina* causative agents of root rot disease in soybean.

MATERIAL AND METHODS

Fungal isolates. Small parts of soybean roots from plants showing yellowing and wilting symptoms were surface sterilised in 5% sodium hypochlorite for 3 minutes. The sterilised fragments were washed several times with sterile distilled water and dried on filter papers. The fragments were assigned to two groups. Fragments of the first group were placed on potato sucrose agar (PSA), while fragments of the second group were placed on Modified Nash and Synders Medium (MNSM) (Cho *et al*. 2001) in Petri dishes of 9 cm diameter. The plates were incubated at 25 ± 2°C for 5 days. *Macrophomina phaseolina* isolates were identified according to Holliday and Punithalingat (1970), and *Fusarium* isolates according to Booth (1981) and stored at 4°C.

Rhizobial isolates. Isolates of *Rhizobium japonicum*, maintained on peat moss, were obtained from Rhizobial Research Laboratory, General State of Applied Research, Ministry of Agriculture, Iraq. The isolates were grown on yeast mannitol broth (YMB) containing 0.1% yeast extract, 1% mannitol, 0.05% potassium phosphate, 0.02% magnesium sulphate, 0.02% NaCl, pH 7.6, in 250 ml Erlenmeyer flasks, in shaking incubator at 28°C for 28 hours. The number of cells/ml in the rhizobial culture was determined by colony count method (Black 1965) on yeast mannitol agar (YMA) and used at 10^8 cells/ml in laboratory and greenhouse experiments.

Rhizobial culture filtrate. Rhizobial filtrate was obtained by centrifugation of rhizobial culture at 6000 rpm for 20 min and the supernatant were passed through 0.45 μm bacterial filter.

Activity of rhizobial culture filtrate against** F. solani **and** M. phaseolina **on culture media. The test was carried out using an agar well diffusion method on PDA culture medium. Four wells of 0.5 cm diameters were equidistantly made on PDA in Petri dishes of 9 cm diameter. One ml of rhizobial filtrate at 0, 25, 50, and 100% was placed separately in each well. A disc of 0.5 cm diameter of fungal growth, from fresh culture on PDA medium, was placed at the centre of the PDA plate. The plates were incubated at 28 ± 2°C for 5 days and the radial growth of fungi was assessed in triplicate Petri dishes. Percentage growth inhibition was calculated according to the following formula: % growth inhibition = (control – treatment/control) × 100.

The efficiency of rhizobial filtrate on the radial growth of the pathogenic fungi was also determined by mixing the filtrate with PDA before solidification at the same above concentrations in Petri dishes. The medium was inoculated by the fungi and the percentage of growth reduction was calculated as before.

Rhizobium antagonism against** F. solani **and** M. phaseolina **in pots. Sterile clay-loamy to peat moss (3:1) soil in pots (2 kg/pot) was contaminated separately by *F. solani*, *M. phaseolina*, and both of them grown on bran and groats at 2 g/kg soil. Each pot was drenched with 10 ml of *R. japonicum* suspension at 1×10^8 cell/ml after 2 days of fungal contamination. Pots contaminated by pathogens only and non-treated ones (without fungal and bacterial inoculant) were considered as control. The pots were arranged in a greenhouse (28 ± 2°C) in a randomised block design in three replicates, cultivated with surface sterilised soybean seeds (10 seed/pot) and watered as needed. The treatments were *F. solani*, *M. phaseolina*, *F. solani* + *M. phaseolina*, *F. solani* + rhizobia, rhizobia only, and control.

The percentage of germination was determined one week after sowing. Woltz and Arther index (Woltz & Arther 1973) was used to determine disease severity. The disease severity was calculated according to the following equation:

$$\text{Disease severity (\%)} = \frac{\Sigma(\text{disease rate} \times \text{number of plant with this rate})}{\text{total number of plants} \times \text{maximum value of disease scale}} \times 100$$

Field trial. Field experiment was conducted in the 2010 growing season on 4/5/2010 at state of Applied Research Station, Baghdad, Abu-Ghraib, in a randomised block design with plots of 3 × 3 m^2 and 3 replicates. Seeds of soybean were dipped in Rhizobium culture suspension at 10^8 cells/ml, containing 2% Arabic gum as sticker, for one hour. Non-treated seed were dipped in distilled water as control. Rhizobium-treated and non-treated seeds were sown in rows 3 m long and 30 cm in spacing.

The fungal inoculum, grown on a mixture of bran and groats, was distributed along the rows at 6 g/m long, 24 h before seed sowing. The treatments were as follows:

(1) Rhizobium treated seeds in *F. solani* contaminated soil;
(2) Rhizobium treated seeds in *M. phaseolina* contaminated soil;
(3) Rhizobium treated seeds in *F. solani* and *M. phaseolina* contaminated soil;
(4) Non-treated seeds in *F. solani* contaminated soil;
(5) Non-treated seeds in *M. phaseolina* contaminated soil;
(6) Non-treated seeds in *F. solani* + *M. phaseolina* contaminated soil;
(7) Non-treated seeds in non-contaminated soil;
(8) Rhizobium treated seeds in non-contaminated soil.

Germination percentage and root rot index were determined as previously after 19 weeks.

RESULTS

Activity of rhizobial culture filtrate against *Fusarium solani* and *Macrophomina phaseolina* on culture medium

Soybean rhizobial culture filtrate showed a significant reduction in the radial growth of *F. solani* and *M. phaseolina* on PDA culture medium. The inhibitory effect of the filtrate on the two pathogens increased with increasing concentration of filtrate. The reduction percentages of the radial growth for the two pathogenic fungi were found to be 33.84, 46.46, 59.27, 65.58% and 39.61, 47.12, 57.06, 64.04% at 25, 50, 75, 100% of rhizobial filtrate, respectively, as determined by the agar well diffusion method (Table 1). On the other side, the reduction percentage of fungal growth by the filtrate was found to be 54.11, 63.33, 67.11, 72.66% for *F. solani*, 46.33, 52.66, 59.66, 73.33% for *M. phaseolina* at the same concentration of rhizobial filtrate, respectively, when determined by a culture poisoning technique.

Effect of Rhizobium seed treatment on seed germination and root rot disease severity under field conditions

Results in Table 2 indicate that the pathogenic fungi, *F. solani*, *M. phaseolina* separately and in combination, affected highly seed germination, 28.5, 35.83, 27.50%, respectively, compared to 60.0% in the control. The coating of soybean seeds with *R. japonicum* increased the germination percentage significantly, 50.93, 56.5, and 50.13% in soil contaminated by *F. solani*, *M. phaseolina* and both, respectively. *R. japonicum* induced a high reduction in disease severity, 36.1, 27.7, 41.6% compared to 83.3, 74.9, and 86.0% on roots for the same previous combination, respectively.

Effect of rhizobial suspension on seed germination and root rot disease development in pots

Results of the effect of rhizobial suspension on seed germination and soybean root rot disease caused by *F. solani* and *M. phaseolina* separately or in combination, in contaminated soil are shown in Table 3. It was found that the two pathogenic fungi caused a high reduction in seed germination. The seed germination percentages were 40.0, 43.3, 46.6% in soil contaminated by *F. solani*, *M. phaseolina* and both, respectively, compared to 76.6% in the control.

The addition of *R. japonicum* suspension to the soil contaminated by the two pathogens improved seed germination and significantly reduced root rot disease severity. The germination percentage was 66.6, 73.3, 63.3% in rhizobial treated soil compared to *F. solani*, *M. phaseolina* and both, respectively.

The reduction of root rot disease severity caused by *F. solani* and *M. phaseolina* in the contaminated

Table 1. Effect of *Rhizobium japonicum* filtrate on the radial growth of *F. solani* and *M. phaseolina* estimated by agar well diffusion method on a culture medium

Filtrate concentration	*Fusarium* radial growth	Inhibition percentage of *Fusarium*	*Macrophomina* radial growth	Inhibition percentage of *Macrophomina*
100	1.8	65.58	2.06	64.04
75	2.3	59.27	2.24	57.06
50	2.8	46.46	3.03	47.12
25	3.46	33.84	3.46	39.61
Control	5.23	0	5.73	0
LSD (*P* = 0.05)	0.355		0.340	

Each value is a mean of 3 replicates

Table 2. Effect of *Rhizobium japonicum* on seed germination and root rot disease severity of soybean

Treatments	Germination (%)	Disease index (%)	
		on foliage	on roots
F. solani	28.50	79.96	83.3
M. phaseolina	35.83	68.83	74.9
F. solani + *M. phaseolina*	27.50	75.53	86.0
R. japonicum + *F. solani*	50.93	46.63	36.1
R. japonicum + *M. phaseolina*	56.50	43.23	27.7
R. japonicum + *F. solani* + *M. phaseolina*	50.13	51.00	41.6
R. japonicum	65.13	30.00	30.5
Control	60.00	30.00	33.3
LSD (*P* = 0.05)	5.06	9.42	16.26

Each value is a mean of 3 replicates

Table 3. Antagonistic activity of *R. japonicum* against *F. solani* and *M. phaseolina*

Treatments	Germination (%)	Disease severity	
		on foliage	on roots
Control	76.6	18.80	9.40
F. solani	40.0	82.20	77.76
M. phaseolina	43.3	50.00	69.40
F. solani + *M. phaseolina*	46.6	68.96	74.96
R. japonicum + *F. solani*	66.6	37.76	44.40
R. japonicum + *M. phaseolina*	73.3	34.43	36.10
R. japonicum + *F. solani* + *M. phaseolina*	63.3	55.53	38.83
R. japonicum	83.3	19.96	27.73
LSD (*P* = 0.05)	12.89	12.49	12.85

Each value is a mean of 3 replicates

soil was highly significant at *P* = 0.05, 37.76, 34.43, 55.53% on foliage with rhizobia, compared to 77.76, 69.4, 74.96% without rhizobia for the two pathogens separately and in combination respectively. On the root system the disease severities were 44.4, 36.1, 38.83% with rhizobia compared to 77.76, 69.4, and 74.96% without rhizobia for the three treatments, respectively.

DISCUSSION

The results of this study revealed that *F. solani* and *M. phaseolina* are among the most important soil borne-pathogens infecting roots and causing up to 40% of plant mortality. The control of soil-borne pathogens is difficult since they produce resting structures such as chlamydospores and sclerotia resistant to adverse environmental conditions. The misuse of chemicals to control these pathogens caused enormous problems to ecosystem and human's health as well as has led to development of resistant races of pathogens (El-Bramawy & Abdul-Wahid 2006; El-Batanony *et al*. 2007).

The present study aims to protect soybean plants against soil-borne pathogens (*F. solani* and *M. phaseolina*) and improve growth and yields by using *Rhizobium japonicum*, an environmentally friendly alternative to fungicides. Results obtained showed that *Rhizobium japonicum* induced a high reduction in fungal radial growth on a culture medium as well as promoted plant growth under greenhouse and field conditions. On the other hand, root rot severity caused by these pathogens was also reduced by the addition of rhizobia to the contaminated soil.

The growth promotion induced by rhizobia may be directly through nitrogen fixation and production of plant growth regulators, i.e. substances that stimulate plant growth. Several researchers reported that rhizobia produced plant growth regulators such as indole acetic acid, auxins, cytokinins, gibberellin-like substances and rhizopine that stimulated and enhanced plant growth (Triplett *et al*. 1981; Atzorn *et al*. 1988; Hussain *et al*. 1990; Sheng 1993; Murphy et al. 1995; Noel *et al*. 1996; Boddey & Hungria 1997; Deshwal et *al*. 2003; Sharif *et al*. 2003). It was also reported that rhizobia increase *P*-availability to plants (De Freitas *et al*. 1997). In addition, the promotion of plant growth may indirectly suppress soil-borne pathogens by rhizobia metabolites. Several previous studies reported that rhizobia increase seed germination significantly and improve plant growth and yields through a reduction of soil-borne pathogens (Sheikh *et al*. 2006; Mazen *et al*. 2008).

The mechanism of rhizobia activity against *F. solani* and *M. phaseolina* may be due to the colonization of plant roots using root exudate for growth and synthesising metabolites protecting the roots against pathogens through antibiosis, degrading pathogenicity and inhibiting spore germination as well as induction of plant defence mechanisms. It was reported that rhizobia in the rhizosphere of plants may prevent the control of pathogenic fungi by covering hyphal tips of the fungus and produce antibiotics leading to the lysis of fungal hyphae (Sharif *et al*. 2003). Several bacterial strains including rhizobia were isolated and used to control soil-borne pathogens, while they showed high efficiency under greenhouse and field conditions (Nelson 2004; Siddiqui 2006; Akhtar & Siddiqui 2009). Rhizobia were reported to inhibit the growth of pathogenic fungi including *F. solani* and *M. phaseolina* in both leguminous and non-leguminous crops (Ehtishamul-Haque & Gaffar 1993; Omar & Abd-Alla 1998).

Besides nitrogen fixation and promotion of plant growth, *Rhizobium japonicum* was found to exhibit high activity against *F. solani* and *M. phaseolina* causative agent of soybean root rot, and could be used as an important element in management of root rot disease.

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