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RESEARCH PAPERS

Induction of resistance in common bean by *Rhizobium leguminosarum* bv. *phaseoli* and decrease of common bacterial blight

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Summary. *Rhizobium leguminosarum* bv. *phaseoli* was evaluated for its capacity to trigger resistance to common bacterial blight (CBB) of common bean caused by *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*), under greenhouse and field conditions. A common bean cultivar and three lines including two tolerant lines (Ks51103 and BF13607) and the susceptible cultivar Khomein and line Ks21479, were used. *R. leguminosarum* bv. *phaseoli*, was applied as a seed treatment and its effect on disease severity (DS) was compared with untreated (control) plants and with urea fertilizer treated plants in both the greenhouse and the field. *R. leguminosarum* bv. *phaseoli* in common bean roots tended to reduce CBB severity and to improve plant growth, particularly the 100-seed weight, in the field. The greatest decrease in CBB in the greenhouse occurred 15 days after inoculation (DAI) of *Xap* in the cultivar Khomein and line Ks21479, and 30 DAI in lines Ks51103 and BF13607. In the field, however, the greatest decrease occurred 35 DAI in the cultivar and all lines. Furthermore, in the field the greatest improvement in 100-seed weight occurred in the cultivar Khomein treated with *R. leguminosarum* bv. *phaseoli*. The potential of using *R. leguminosarum* bv. *phaseoli* in the management of CBB and the possible mechanisms that induce resistance in this symbiotic system are discussed.

Key words: *Xanthomonas axonopodis* pv. *phaseoli, Phaseolus vulgaris*, Iran.

Introduction

Common bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*), is a major seed-borne disease of common bean (*Phaseolus vulgaris* L.) worldwide (Tar'an *et al.* , 2001; Miklas *et al*., 2003; Mkandawire *et al*., 2004) and causes losses in yield from 10 to 40% (Opio *et al*., 1996). In Iran, CBB was first reported from Markazi province (Lak *et al*., 2002). Recently, CBB has become a major disease causing great loss in yield. In the past few years, the causal agent of the disease has spread to neighboring provinces, including Lorestan, Isfahan and ChaharMahal-va-Bakhtiari (Zamani, 2008). In many countries seeds planted by farmers are already infected with CBB (Coyne *et al*., 2003). CBB is difficult to control as field spraying with bactericides is ineffective and uneconomical (Zanatta *et al*., 2007). The best means to control CBB is by using pathogen-free seed (Zanatta *et al*., 2007) and planting CBB-resistant cultivars (Miklas *et al*., 2003; Asensio-S.-Manzanera *et al*., 2006). Cultural practices such as crop rotation, weed elimination and removal of

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plant debris also contribute to the control of CBB (Saettler, 1991).

Rhizobium leguminosarum bv. *phaseoli* is a bacterium that establishes a symbiotic relationship with common bean within the root nodules, where atmospheric nitrogen is fixed into a form useful for plant growth (Jordan 1984; Paul and Clark 1989; Long 2001) a relationship which often improves crop yield. The practice of inoculating common bean and other legumes grown under nitrogen deficiency with *Rhizobium* spp. strains exhibiting high levels of nitrogen fixation can increase yield (Schallmach *et al*., 2000). The success of the symbiosis, reflected in enhanced yield, depends largely on the performance of the nodulating rhizobia (Mrabet *et al*., 2005).

Certain strains of *Rhizobium* spp. are effective biocontrol agents for the control of diseases of common bean. There are reports on the potential of *Rhizobium* spp. strains (Jensen *et al*., 2002; Huang *et al*., 2007; Zanatta *et al*., 2007; Akhtar and Siddiqui 2008) and other antagonistic bacterial strains (Peix *et al*., 2001) to manage diseases of common bean and other plants (Reitz *et al*., 2000; Arora *et al*., 2001). Using an mRNA differential display for healthy and Fusarium-infected roots of common bean, Lange *et al*. (1999) isolated a common bean cDNA encoding a putative receptor-like protein kinase (RLK), designated *PvRK20-1* that is induced during pathogen attack. Levels of *PvRK20-1* mRNA increased in roots treated with purified Nod factors from *R. tropici*, whereas Nod factors from the non-host *Rhizobium* spp. strain NGR234 had only slight effect (Lange *et al*., 1999). This suggests that *Rhizobium* spp. may induce resistance in common bean and limit CBB. Furthermore, there are similarities between plant responses to symbiotic rhizobia and to pathogens. Bartsev *et al*. (2004a, b) found that when the nodulation outer protein L (NopL) from *Rhizobium* spp. was delivered into plant cells, it nodulated the activity of the signal transduction pathways that culminated in the activation of PR proteins. In *Glycin max* roots, *Bradyrhizobium japonicum* Nod factors induce chitinase CH1. Plant chitinases are usually induced during pathogen attack suggesting that they play a role in plant defense (Métraux and Boller 1986; Xie *et al*., 1999).

The main purpose of this study was to determine if *R. leguminosarum* bv. *phaseoli* has a role in decreasing or inhibiting CBB in resistant and susceptible common bean lines under greenhouse and field conditions.

Materials and methods

Plant materials

Based on the findings of a prior study (Osdaghi *et al*., 2009a), one CBB-susceptible cultivar and one susceptible line (cv. Khomein and line Ks21479) and two CBB-tolerant lines (Ks51103 and BF13607) were selected for greenhouse and field study. The cultivar and all the lines were obtained from the National Center for Bean Research in Khomein, Markazi province, Iran, and they were certified free of CBB and other contaminants by the National Center for Bean Research. The characteristics of the cultivar and lines were described in an earlier study (Osdaghi *et al*., 2009a).

Bacterial strains

Xanthomonas axonopodis *pv.* phaseoli

The Araxal strain of *Xap* was used in the study. The biochemical characterization and the PCR based identification of this strain were done as described by Schaad *et al*. (2001) and Audy *et al*. (1994) respectively. The isolate was identified as xanthomonad-like based on yellow, convex, mucoid colony morphology, and on biochemical characteristics: gram negative reaction, hypersensitive reaction on tobacco, potato soft-rotting negative, levan positive, inhibition of growth by 2.5% NaCl, catalase positive, oxidase weak, gelatin hydrolysis positive (Schaad *et al*., 2001), and it was further confirmed as *Xap* by amplifying a 700-bp DNA fragment with the X4c/X4e primer pair (Audy *et al.,* 1994). Descriptive information of Araxa1 has previously been published elsewhere (Osdaghi *et al*., 2009a). The pathogenicity of this strain on the cv. Khomein was also demonstrated in a previous study (Osdaghi *et al*., 2009a).

R. leguminosarum *bv.* phaseoli *strain*

Rhizobium leguminosarum bv. *phaseoli* was obtained from the Institute of Soil-Water Research, Tehran, Iran. This strain is reported to form root nodules on common bean. The effectiveness of this strain in improving plant growth parameters such as dry shoot and root weights and the number of pods per plant has recently been reported (Osdaghi *et al*., 2009b).

Greenhouse experiment

The experiment was carried out in a randomized complete block design consisting of two pots per treatment and three plants per pot with three replications (eighteen plants per treatment). For both *Xap* and *R. leguminosarum* bv. *phaseoli* isolates, 48 h old bacterial cultures on nutrient agar (NA) were suspended in distilled water and adjusted to approximately 10^8 cfu mL^{-1}. Each bacterial suspension was applied following a suitable method (Abeysinghe, 2007). Two treatments were applied: 1, seeds were soaked in *R. leguminosarum* bv. *phaseoli*; 2, plants were treated with urea fertilizer (urea 46% granule). Control seeds were dipped in distilled water. Seeds from the cultivar and the lines were surface-sterilized in 20% sodium hypochlorite for five minutes, washed with distilled water twice, soaked in *R. leguminosarum* bv. *phaseoli* suspension for 1 h, and planted in 20 cm diameter plastic pots containing a mixture of perlite, soil and peat (1:1:1 v:v:v) with five seeds per pot, thinned to three uniform seedlings per pot after one week (Daza, 2000). Plants were fertilized with a urea suspension (50 mg per pot) when they were at the germination stage.

Fifteen days after germination, seedlings were inoculated with a log-phase culture of *Xap*, as described in an earlier study (Osdaghi *et al*., 2009a). Control plants received distilled water at the same rate. Experiments were conducted under natural light in a greenhouse with a 16 h day and day/night temperatures of 26/22°C.

Symptoms of CBB were scored using the following scale: 0, symptomless, 1, negligible symptoms or slight marginal necrosis, 2, water-soaking, chlorosis or necrosis (blight) in <25% of the inoculated area, 3, 25–50% blight 4, 50–75% blight and 5, complete necrosis of the leaves (Dursun *et al*., 2002; Lak *et al*., 2002; Osdaghi *et al*., 2009a). In the greenhouse experiment disease severity (DS) was assayed at 10, 15, 20 and 30 days after inoculation (DAI). The greenhouse experiment was conducted twice and all eighteen plants in each treatment were inspected for DS in both replications.

Field experiment

To determine the effect of *R. leguminosarum* bv. *phaseoli* in the roots on CBB severity in the leaves under natural field conditions, a field experiment was conducted at the Center for Agricultural Research in Markazi Province, Arak, Iran in May 2008. The experiment was conducted in a randomized complete block design with three replications (separate blocks). Two treatments were applied: 1, inoculation with *R. leguminosarum* bv. *phaseoli*, 2, treatment with urea fertilizer (250 kg h^{-1} of 46% urea). Control plants received neither *R. leguminosarum* bv. *phaseoli* nor urea fertilizer. The suspension of *R. leguminosarum* bv. *phaseoli* was prepared as above. *R. leguminosarum* bv. *phaseoli* was inoculated in two steps as follows. First, surface disinfected seeds of common bean were soaked in a bacterial suspension and planted, secondly, three days after the seedlings germinated, they were treated with the same bacterial suspension. Urea fertilizer was applied to the soil twice, when the plants planted and again when they began flowering. Each treatment was given in a field plot 4 m long and 3 m wide. Seeds were planted in five rows per plot. Seedling density was adjusted to 50 cm × 10 cm. Plants were watered by a sprinkler system as needed. The inoculum of *Xap* was prepared as described for the greenhouse experiment. Plants were sprayed with a suspension containing about 1×10^8 cfu mL^{-1} delivered by a pressure sprayer at the time of flowering. Prior to inoculation plants were sprayed with water to provide a favorable microclimate for bacterial infection (Webster *et al*., 1983). In the field experiment, the severity of CBB was scored 20, 35 and 50 DAI by *Xap*. The 100-seed weight was also measured. DS was scored for at least 15 plants per plot.

Statistical analysis

For each of the greenhouse and field experiments, DS index was subjected to analysis of variance (ANOVA; P=0.05). Duncan's multiple range tests were used to separate the treatment means. Repeats of the same experiment did not differ in either the greenhouse or the field. Results of each experiment were therefore combined and analyzed together. MSTAT-C computer software was used for the statistical analysis (Russell and Eisensmith, 1983).

Results

Greenhouse experiment

In the greenhouse experiment, CBB symptoms

Table 1. Analysis of variance for CBB severity in a *R. leguminosarum* bv. *phaseoli*-common bean symbiosis greenhouse experiment, 10, 15, 20 and 30 days after inoculation of *Xap* on common bean plants.

Source of variance	DF[a]	Mean of squares			
		10 DAI	15 DAI	20 DAI	30 DAI
Path	1	134.173**	169.000**	254.668**	437.506**
Rhi	2	2.548**	3.062**	4.064**	6.131**
Line	3	10.395**	11.671**	15.404**	14.456**
Path × Rhi	2	2.548**	3.062**	4.064**	6.131**
Rhi × Line	6	0.854**	0.594**	0.203**	0.365**
Path × Line	3	10.395**	11.671**	15.404**	14.456**
Path × Line × Rhi	6	0.8541**	0.594**	0.203**	0.365**
Error	120	0.031	0.046	0.060	0.065
CV		18.51	19.91	18.53	14.65

[a] DF, degree of freedom.

**, Significant at $P<0.01$.

appeared 10–12 DAI, but the DS varied depending on the lines and treatments. Control plants never exhibited CBB symptoms. Symbiotic nodules were seen on plants inoculated with *R. leguminosarum* bv. *phaseoli* but not on plants treated with urea fertilizer or on control plants. Statistical analysis of both replications of the greenhouse experiment showed that the results of these replications were the same. The data of these experiments were therefore combined (Table 1). Significant differences were seen in the DS between plants inoculated with *R. leguminosarum* bv. *phaseoli,* urea fertilized plants and control plants ($P<0.05$). DS was scored 10, 15, 20 and 30 DAI. At 10 and 15 DAI, the DS differed significantly between treatments only in the susceptible group cv. Khomein and line Ks21479 ($P<0.01$) (Figure 1A and B: a1b1, a1b2, a1b3, a2b1, a2b2 and a2b3 bars). In the tolerant group (lines Ks51103 and BF13607) on the other hand, the DS did not differ significantly between the treatments (Figure 1A and B: a3b1, a3b2, a3b3, a4b1, a4b2 and a4b3 bars) and in the control plants the CBB symptoms were negligible or consisted only in a slight marginal necrosis.

At 20 DAI, in the tolerant lines BF13607 and Ks51103, the DS did not differ significantly between plants inoculated with *R. leguminosarum* bv. *phaseoli* and control plants (Figure 1C: a3b1, a3b2, a4b1 and a4b2 bars respectively), but the DS in the urea fertilized plants was significantly greater than that in the others (Figure 1C: a3b3 and a4b3 bars respectively). At this time the DS of both the susceptible cultivar Khomein and line Ks21479, differed significantly between inoculated plants and urea-fertilized plants ($P<0.01$).

When plants in the greenhouse, were scored for DS for the last time, (30 DAI), the susceptible cultivar and line and the tolerant lines differed significantly in their DS ($P<0.01$). Although *R. leguminosarum* bv. *phaseoli* significantly lowered the DS in the cultivar and 3 lines ($P<0.01$), it reduced the DS more in the tolerant lines (Figure 1D). Moreover, the effect of *R. leguminosarum* bv. *phaseoli* and urea fertilizer on some yield parameters such as plant dry shoot and root weights, the number of pods per plant and the number of seeds per pod, was also evaluated at 40 DAI (data not shown). This evaluation showed that *R. leguminosarum* bv. *phaseoli* and urea fertilizer were equally effective in improving these parameters.

Field experiment

In the field experiment, CBB symptoms were inspected 18–20 DAI. The symptoms in the field experiment were similar to those of the greenhouse only once (Table 2). At 20 DAI, the DS differed significantly between the *R. leguminosarum* bv. *phaseoli* inoculated plants, the urea fertilized

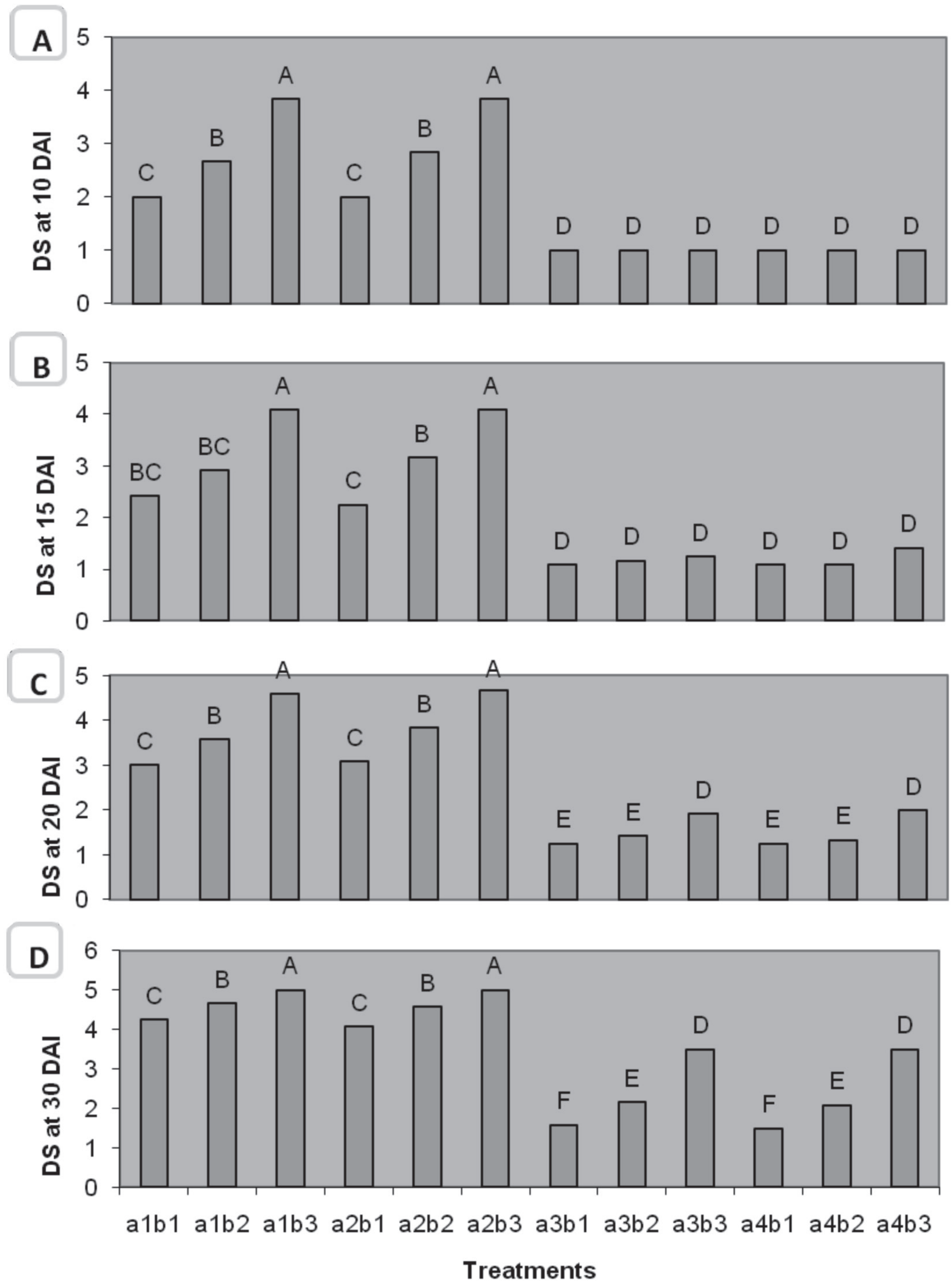

Figure 1. Effect of *Rhizobium leguminosarum* bv. *phaseoli* in the roots of common bean plant on disease severity (DS) of common bacterial blight (CBB) under greenhouse conditions 10, 15, 20 and 30 days after inoculation with the pathogen *Xanthomonas axonopodis* pv. *phaseoli*. "a": cultivar and lines including: a1, cultivar Khomein; a2, line Ks21479 (CBB-susceptible group); a3, line BF13607 and a4, line Ks51103 (CBB-tolerant group). "b": different root treatments including: b1, inoculation with *R. leguminosarum* bv. *phaseoli*; b2, control; and b3, fertilization with urea. Different letters above bars indicate significant statistical differences between treatment groups ($P<0.05$).

Table 2. Analysis of variance for 100-seed weight and CBB severity evaluation in a *R. leguminosarum* bv. *phaseoli*-common bean symbiosis field experiment, 20, 35 and 50 days after inoculation of *Xap* on common bean plants.

Source of variance	DF[a]	Mean of squares			
		20 DAI	35 DAI	50 DAI	wt. of 100 seeds
Rep	2	0.299	0.049	0.090	1.825
Rhi	2	6.674**	8.799**	6.924**	67.777**
Line	3	6.081**	9.192**	11.025**	614.711**
Line × Rhi	6	0.248	0.095**	0.942**	13.907**
Error	22	0.102	0.124	0.037	2.177
CV		15.20	11.49	5.24	4.74

[a] See Table 1.

**, See Table 1.

plants and the control plants ($P<0.05$) for the cultivar and all lines except the tolerant line BF13607 where the DS of *R. leguminosarum* bv. *phaseoli* inoculated plants and control plants was similar (Figure 2A: a3b1 and a3b2 bars). Nonetheless, the DS in urea-fertilized plants was significantly higher in BF13607 (Figure 2A: a3b3 bars). At 35 DAI, too, the DS differed significantly between the treatments in the cultivar and all lines ($P<0.05$) (Figure 2B). The final DS in the field was recorded 50 DAI. At this time CBB was very sever (complete necrosis of leaves) in the susceptible cultivar and line that were urea fertilized and that were in the control group, with very severe loss in yield (data not shown), but in the plants inoculated with *R. leguminosarum* bv. *phaseoli* the DS was still relatively low ($P<0.01$). Yield loss was evaluated by comparing the 100-seed weight of each group of plants. The 100-seed weight of the urea-fertilized plants was lower than that of the control plants and also than that of the plants inoculated with *R. leguminosarum* bv. *phaseoli* (data not shown). On the other hand, the quality of the seeds from urea fertilized plants (seed coat lesions and seed size) was lower than that of seeds from the other plants. This is taken to be a negative effect of urea fertilizer on plant yield. Furthermore, the reduction in CBB symptoms of the cv. Khomein was even greater than that in line Ks21479 (Figure 2C: a1b1 and a2b1 bars). At 50 DAI, the tolerant BF13607 line, has the highest DS when it was urea fertilized and the lowest DS when it was inoculated with *R. leguminosarum* bv. *phaseoli*.

Unlike the DS at the other recording dates, at 50 DAI the DS of line Ks51103 inoculated with *R. leguminosarum* bv. *phaseoli* was greater than that of the control plants (Figure 2C: a4b1 and a4b2 bars), whereas, the DS of the urea fertilized plants was significantly greater than in the inoculated or the control plants (Figure 2C: a4b3 bar). In cultivar Khomein and lines Ks21479 and BF13607 of the field experiment the 100-seed weight differed significantly between the two treatments and control ($P<0.01$) (Figure 3). In the cultivar and all lines the highest 100-seed weights occurred in plants inoculated with *R. leguminosarum* bv. *phaseoli*, and the lowest in urea fertilized plants. In the CBB-tolerant line Ks51103, on the other hand, the highest 100-seed weight occurred in plants inoculated with *R. leguminosarum* bv. *phaseoli* and the 100-seed weight of the urea fertilized plants was greater than that of the control plants ($P<0.05$). These findings and those on the DS at 50 DAI in the field experiment indicated that in line BF13607 the positive effect of *R. leguminosarum* bv. *phaseoli* was greater than that in line Ks51103.

Discussion

The objective of the work was to study the effect of *R. leguminosarum* bv. *phaseoli*, a symbiont on common bean on the development of CBB under greenhouse and field conditions. Both greenhouse and field experiments showed that inoculation of both the CBB-susceptible cultivar and line (cultivar Khomein and line Ks21479) and the CBB-tolerant lines BF13607 and Ks51103 with *R.*

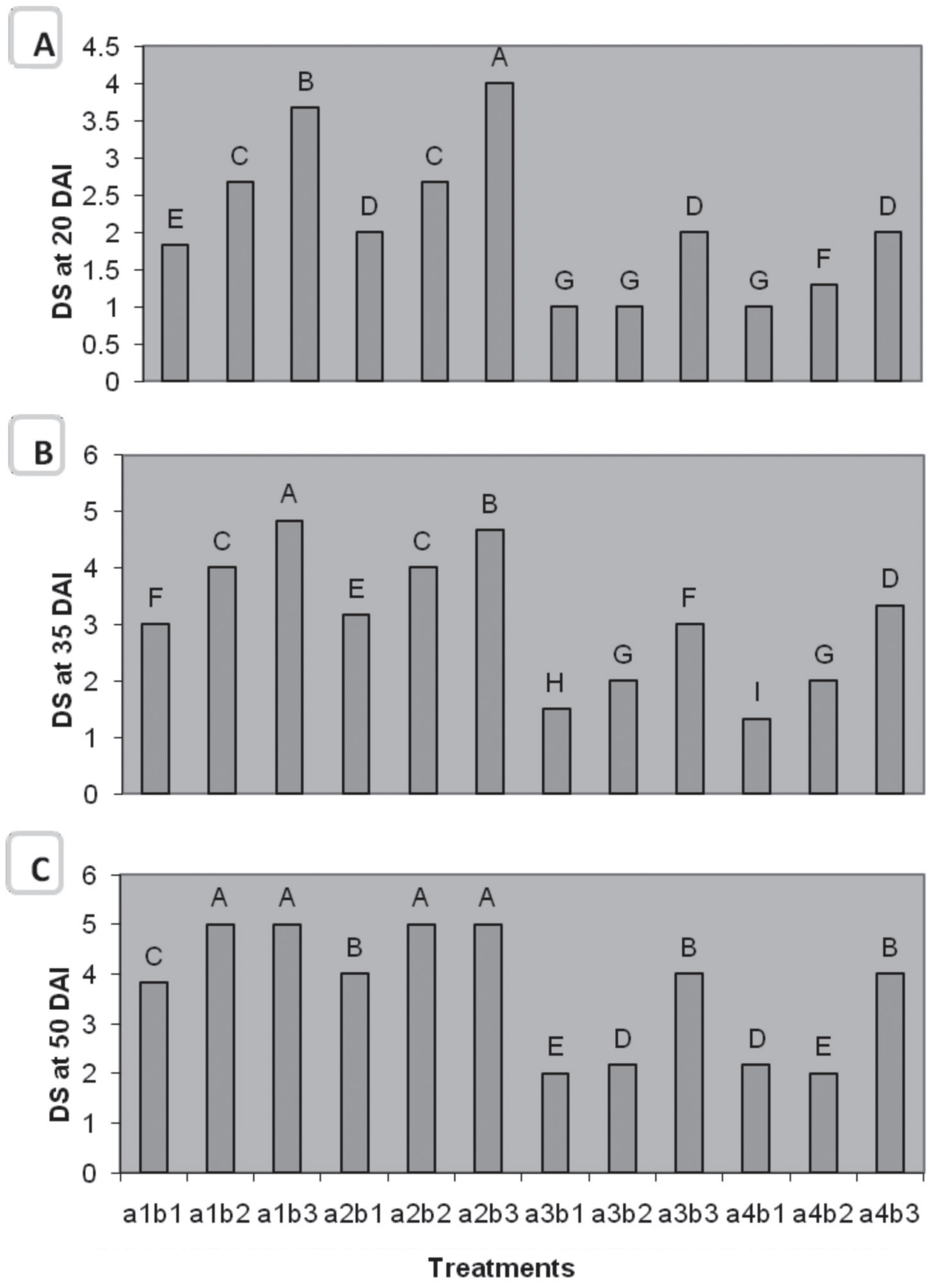

Figure 2. Effect of *Rhizobium leguminosarum* bv. *phaseoli* inoculated in common bean roots on common bacterial blight (CBB) disease severity (DS) in field conditions 20, 35 and 50 days after inoculation of *Xanthomonas axonopodis* pv. *phaseoli*. For abbreviations see Figure 1.

leguminosarum bv. *phaseoli* significantly reduced the DS of CBB ($P<0.05$).

Since the commercial bactericides applied in common bean fields do not adequately control CBB and are not economically justifiable (Zanatta *et al.*, 2007), any method that reduces CBB at low costs and that is environmentally safe is of great importance. As has been mentioned, none of the common bean cultivars or lines planted in Iranian fields is resistant to CBB (Osdaghi *et al.*, 2009a) and it is advisable to select CBB-tolerant lines. As shown in this study, *R. leguminosarum* bv. *phaseoli* in common bean roots improves the tolerance of common bean lines to CBB. To our knowledge, this is the first report that a symbiotic relationship between a legume plant and a rhizobial bacterium reduces the foliar symptoms of a disease.

Rhizobium leguminosarum bv. *phaseoli* and urea fertilizer improved growth parameters such as plant shoot dry weight, root weight, number of pods per plant and number of seeds per pod (Osdaghi *et al*., 2009b) and the symbiotic relationship between this bacterium and common bean decreased CBB severity in both the susceptible cultivar and in tolerant lines. However, adding urea fertilizer to the soil increased disease severity (Figures 1 and 2).

In the greenhouse experiment, at 10 DAI, the susceptible cultivar Khomein and line Ks21479 differed significantly in DS of CBB between plants inoculated with *R. leguminosarum* bv. *phaseoli,* plants fertilized with urea and control plants, whereas, the tolerant lines BF13607 and Ks51103 did not differ between these treatments either 10 or 15 DAI (Figure 1A and B). These findings suggest that disease development in CBB-tolerant plants was not affected by *R. leguminosarum* bv. *phaseoli* or by urea fertilizer until 15 DAI. But at 20 and 30 DAI, significant differences in DS between the three treatment groups of both the susceptible cultivar and lines and the tolerant lines appeared (Figure 1C and D). In all lines and all treatments or all DAI except those mentioned above, the DS of plants inoculated with *R. leguminosarum* bv. *phaseoli* was lower than that of control plants and was also lower than the DS in the plants fertilized with urea.

In the greenhouse experiment, in the last measurement of the DS 30 DAI, *R. leguminosarum* bv. *phaseoli* was more effective in reducing CBB symptoms in tolerant lines than it was in reducing them in the susceptible cultivar and lines (data not shown). Souza *et al*. (2000) reported that four QTLs were associated with both the efficiency of *Rhizobium*-plant symbiosis and its resistance to CBB, suggesting a common genetic control of the response to bacterial inoculation in common bean. Our findings confirmed that *R. leguminosarum* bv. *phaseoli* reduced CBB more on tolerant lines than on susceptible lines.

In the field experiment, although the number of pods per plant and the number of seeds per pod (data not shown) were similar between urea fertilized plants and *R. leguminosarum* bv. *phaseoli* inoculated plants, differences in the 100-seed weight between these groups implied that *R. leguminosarum* bv. *phaseoli* had a positive effect on CBB suppression (Figure 3). Contamination of common bean pods and seeds with *Xap* lowers seed quality and reduces seed weight (Grum *et al*., 1998; Fininsa and Tefera, 2001). In the field experiment, urea fertilizer led to sever CBB in the shoots as well as in the pods and seeds, which reduced the 100-seed weight, whereas, inoculation with *R. le-*

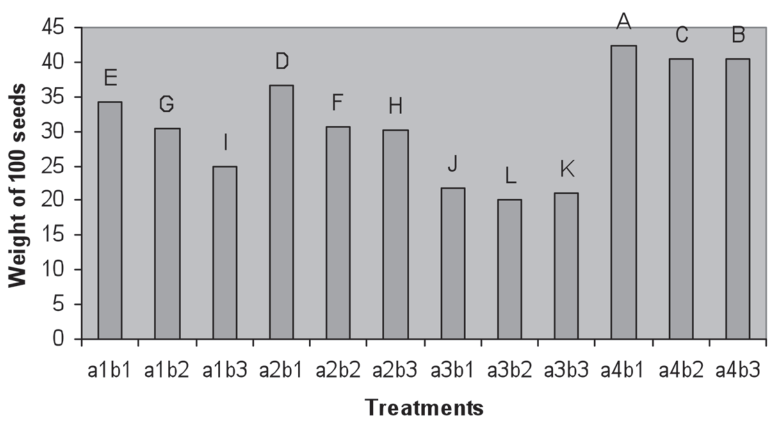

Figure 3. An hundred-seed weight of common bean plants inoculated with *R. leguminosarum* bv. *phaseoli* or fertilized with urea in field conditions. For abbreviations see Figure 1.

guminosarum bv. *phaseoli* produced a high 100-seed weight compared with both the control and especially the urea fertilized plants, lowering the severity of CBB.

In conclusion, since in Iran the applications of nitrogenous fertilizers to common bean is conducted on a tentative basis and there are no standard criteria or standard recommendations, there is always a risk of applying too much or too little nitrogen to the soil. Moreover, Dietrich *et al*. (2004) who were confirmed by the present study, found that both a deficiency and an excess of nitrogen in the soil may render plants more susceptible to disease. The effects of co-inoculation of *R. leguminosarum* bv. *phaseoli* and *Xap* on common bean (Tsai *et al*., 1998; Souza *et al*., 2000; Tar'an *et al*., 2001) and the results of this study, provide evidence for the belief that inoculation of common bean with adaptable strains of *Rhizobium* spp. improves tolerance against CBB and helps manage CBB in common bean fields. Although further proof is necessary to corroborate the effectiveness of *R. leguminosarum* bv. *phaseoli* in reducing the DS of CBB, the use of this bacterium in common bean planting areas in Iran is recommended.

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