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EFFECT OF *Bacillus licheniformis* (BLI008) ON ROOT ROT DISEASE and GROWTH OF CHILLI

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ABSTRACT

***Bacillus licheniformis* isolate BLI008 from a citrus groove in Malaysia was evaluated for its ability to suppress root rot of chilli (*Capscicum annuum*) caused by *Rhizoctonia solani*. Results of the pot experiment revealed that the bacterial antagonist reduced root rot of chilli when seedlings were raised in inoculated (10^9 CFU/ml) nursery medium. Shoot and root growth, plant biomass and leaf chlorophyll content increased significantly ($p<0.05$) with bacterial inoculation in both *R. solani* inoculated and un-inoculated soils. Growth stimulation effect was more pronounced during the early stages of growth. Bacteria inoculated chilli plants produced early flowering and significantly higher number of flowers ($p<0.05$). Therefore, this study suggests that the bacterial isolate BLI008 is an effective agent in the biological control of chilli seedling damping off and root rot caused by *R. solani* and its plant growth enhancement ability could be considered as an added advantage.**

KEYWORDS: *Bacillus licheniformis* BLI008, Chilli, damping off, root rot, chlorophyll

INTRODUCTION

Chilli (*Capsicum annuum*) is one of the important vegetables and spice crops cultivated all over the world. It is ranked the first and third most important vegetable in Asia and the world, respectively, in terms of area cultivated. The production and consumption of chilli have steadily increased worldwide during the 20th century (Crosby, 2008). However, root rot or wilt disease caused by *Rhizoctonia solani* is one of the serious threats of chilli cultivation in most humid areas of the world (Bosland and Votava, 2000; Rini and Sulochana, 2006). *Rhizoctonia solani* is a ubiquitous pathogenic fungus that is known to infect over 500 plant species (McGovern *et al.*, 2002). The pathogen infects chilli primarily through its roots, and the losses attributed to this infection is enormous, which are in the form of seed rot, seedling damping-off resulting poor nursery stands, and reduced yield as a result of infection especially after transplanting (Sanogo, 2003; Rini and Sulochana, 2006). Chilli cultivars commercially available have no resistance to *R. solani* (Crosby, 2008), and only limited partially resistant wild *Capsicum*

germplasms are available in the world (Muhyi and Bosland, 1995). The control of Rhizoctonia diseases is mainly achieved by using fungicides, due to difficulty of using cultural methods such as rotation and tillage to reduce the inoculum of the pathogen (Henis *et al.*, 1978). However, environmental pollution, and development of resistance to the fungicides due to large scale utilization of chemical fungicides (Gerhardson, 2002) has urged research on alternative ways such as biological control to fight against the pathogen. Biological control is a method that is economical and most importantly sustainable to achieve the goal of increasing crop yield (Cook and Baker, 1983). *Bacillus licheniformis* have shown its capabilities in managing several plant pathogenic fungi (Zouaoui *et al.*, 2008; Lim, 2009). Although there is information on the use of this species against other diseases, information on its effect on root rot disease and chilli growth has not been reported. The objective of this is study was to ascertain the potential of *Bacillus licheniformis* in controlling chilli damping off and root diseases caused by *R. solani* and its effect on chilli plant growth.

MATERIALS AND METHODS

Antagonistic bacterial preparation *Bacillus licheniformis* BLI008 (originally isolated from the citrus groove at Tambun, Perak, Malaysia), and pathogenic fungus *Rhizoctonia solani*, were obtained from the culture collection of the Plant Pathology Laboratory of the University Putra Malaysia. The fungal and bacterial isolates were maintained at room temperature (28±2°C) by repeated sub culturing on Potato Dextrose Agar (PDA) and Nutrient Agar (NA), respectively. To prepare the inoculum, a 0.5 cm diameter agar plug from the periphery of two days old culture of *R. solani* on PDA was used. It was transferred into sterilized potato sand medium consisted of 10 % peeled and chopped potato + 90 % sand + 15 % distilled water by weight. The mixture (250 g) was incubated for two weeks at room temperature in 500 ml Erlenmeyer flasks (modified from Ownley and Windham, 2008) before inoculating the potting media.

Chilli (*Capsicum annuum* var. "Kulai") seeds obtained from the Commercial Unit of Department of Agriculture, Malaysia, were surface sterilized for 1 min in 70 % ethanol followed by rinsing five times in sterile distilled water (Szczech and Shoda, 2004) before use. These seeds were sown in multi-celled plastic trays (104 cells per tray; one seed per cell) filled with sterilized peat soil. One tray was treated with BLI008 at a rate of 3 ml (1×10^9 CFUs/ml) suspension per cavity (Szczech and Shoda, 2004) immediately after seeding. One tray was maintained untreated. Six weeks old seedlings raised in these trays were used for transplanting into plastic pots (20 cm x 20 cm x 20 cm) filled with soil. Nitrophoska Green® (15:15:15:2- N: P_2O_5: K_2O: S) was added at the rate of 15 g per pot at one week before *R. solani* inoculation. The *Rhizoctonia* inoculum prepared as described earlier was mixed with the soil at

the rate of 2 % inoculums by weight (120 g/pot) and incubated for seven days in a greenhouse. Chilli seedlings were transplanted in pots at 7 days after inoculation. Watery suspension of BLI008 at the rate of 500 ml (1 x 10^9 CFUs/mL) per pot was added at 4 and 7 days before and 4 days after transplanting and on the day of transplanting as described in Table 1.

Table 1. Timing of application of antagonistic bacterial preparation (BLI008) to potting media in different treatments

Treatment	Description
T1	Chilli seedlings raised in sterilized peat soil was transplanted in *R. solani* inoculated potting medium which was drenched with BLI008 at four days before transplanting (4DBTP)
T2	Chilli seedlings raised in sterilized peat soil was transplanted in *R. solani* inoculated potting medium which was drenched with BLI008 at the time of transplanting (0TP)
T3	Chilli seedlings raised in sterilized peat soil was transplanted in *R. solani* inoculated potting medium which was drenched with BLI008 four days after transplanting (4DATP)
T4	Chilli seedlings raised in sterilized peat soil but inoculated with BLI008 was transplanted in *R. solani* inoculated potting medium 7DBTP
T5	Chilli seedlings raised in sterilized peat soil was transplanted in *R. solani* inoculated potting medium 7 DBTP (pathogenic effect control)
T6	Chilli seedlings raised in sterilized peat soil was transplanted in potting medium inoculated only with BLI008, at the time of transplanting (beneficial effect control)
T7	Chilli seedlings raised in sterilized nursery medium was transplanted in potting medium (without *R. solani* or BLI008 inoculation- un-inoculated control)

Treatments were arranged in CRD with 8 replicates, each represented by a single plant. Six weeks old chilli seedlings transplanted in pots (one seedling per pot) were kept in a greenhouse maintaining constant soil moisture by daily watering. Destructive sampling was done at 30 days after transplanting (DAT) to measure root disease severity and vegetative growth parameters. After 75 days, other four remaining plants were destructively sampled to measure root diseases and vegetative growth. Roots were carefully washed under running tap water and the severity of root rot was assessed. The scale used for rating the severity of root symptoms was from 0 to 4 where, 0= healthy, 1=necrotic rootlets or lesions on primary roots, 2=necrotic secondary or primary roots, 3=necrotic secondary or primary roots with collar rot, 4=collapsed roots with no living tissues, and collar rot (modified from Café-Filho and Duniway, 1995). The Root Disease Severity (RDS) % was estimated using the Equation 1;

$$RDS\ \% = (\Sigma\ (x_i\ n_i)/XN)100 \text{ ---------- Eq 1}$$

where, X_i represents disease severity grade based on 0-4 scale, n_i indicates the number of diseasesd plants on the i^{th} grade of disease scale, N represents totall

number of plants evaluated and X is the maximum disease rating (Cardoso *et al.*, 2004). The treatments T6 and T7 was excluded form the analysis because both treatments were free from *R. solani*.

Plant height (cm), shoot (leaves + stem) dry weight (g), root length (cm) and root dry weight (g) were measured. Plant height was measured from the soil level to the apical bud, while root length was measured from the collar of the transplant to the tip of the longest root. Plants were excised at the soil level to separate shoots and roots, oven dried at 80°C for 48 hrs to constant weight (Mena-Violante and Olalde-Portugal, 2007), and dry weights were determined. The percentage of increase/decrease of shoot and root growth, and total biomass (shoot + root dry weight) were compared with untreated control (T7).

The date of flowering and the number of flowers produced up to 75 DAT was recorded. Leaf chlorophyll content was determined by using samples from 30 day old transplants (Hariprasad *et al.*, 2009).

Statistical significance of the data was determined by ANOVA procedure using SAS computer software version 9.2. Significance of treatment effects was determined by *F*-test ($p=0.05$) and treatment means were compared by the DMRT.

RESULTS AND DISCUSSION

Seedlings raised in bacterized nursery medium (T4), or inoculated in different occasions (T1, T2, T3) showed significant ($p=0.05$) inhibition of *Rhizoctonia* disease expressed as root and collar rot (Figure 1). Observations made 30 DAT revealed that inoculation at sowing (T4) significantly reduced RDS averaging about 6.5 %, whereas RDS in T1 and T2 averaged about 19 % and 24 %, respectively. Treatment T3 showed higher RDS than the other three, averaging about 37.5 %. The highest RDS was observed in T5 (87.5 %), where all the plants died 55 DAT. At 75 DAT, the RDS of all treatments was higher than that of 30 DAT (Figure 1). The BLI008 applied as a soil drench was not effective in inhibiting *Rhizoctonia* root rot at 75 DAT (T1, T2 and T5) but a satisfactory level of disease suppression was achieved in T4. The RDS observed 75 DAT in T1, T2, and T3 were 25 %, 32 % and 44 %, respectively, but it was significantly lower in T4 (9 %; $p<0.05$) (Figure 1).

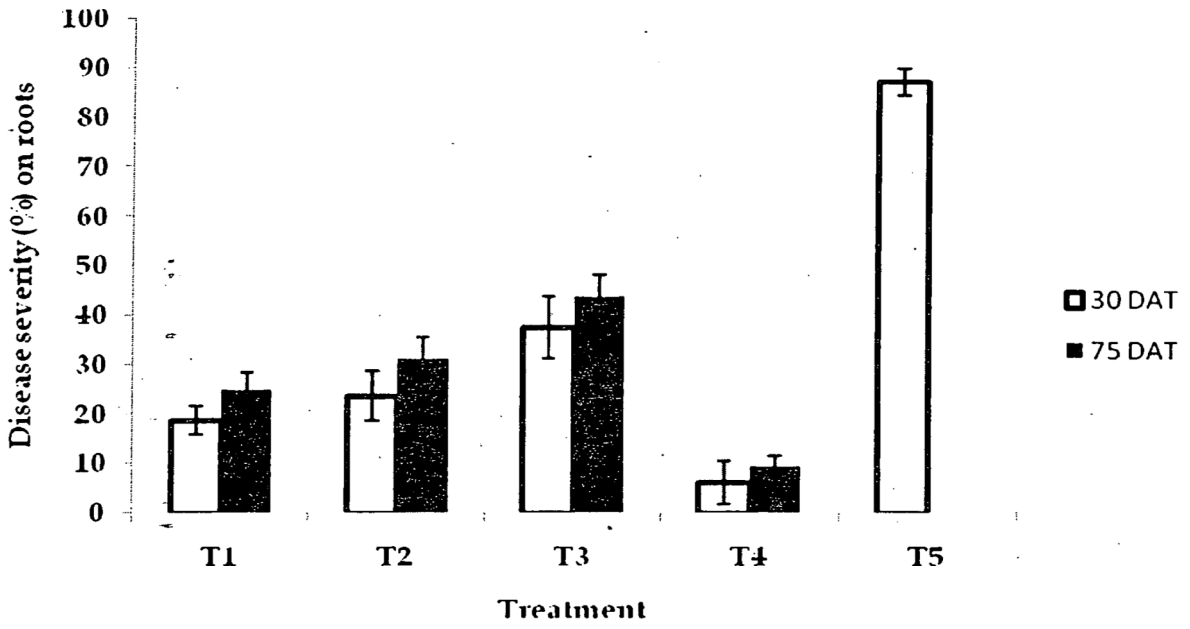

Figure 1. Mean RDS% of chilli plants grown in pots inoculated with (T1, T2, T3, T4) or without BLI008 (T5) at 30 and 75 DAT. Vertical bars represent means±SE. No plant survived up to 75 days in T5. Treatments T6 and T7 were excluded form the analysis because both treatments were free from *R. solani*.

The effect of different treatments on the growth parameters and leaf chlorophyll content is shown in Table 2. 3 and Figure 2, respectively. Inoculation with BLI008 significantly enhanced plant growth and leaf chlorophyll content of chilli plants in pathogen-free soil. BLI008 increased plant height by 26.2 %, root length by 33.9 %, and total biomass by 173.5 % compared to the control at 30 DAT. This growth promotion decreased towards maturity, suggesting that BLI008 mediated positive effects are more pronounced during the early stage of growth.

Days to first flowering and the number of flowers per plant showed significant differences between treatments applied (Table 4). Plants grown in pathogen-free soil but inoculated with BLI008 registered early flowering (48.5 days) and significantly ($p<0.05$) greater number of flowers per plant (27.7 than the untreated control). The results showed that inoculation of growth medium with BLI008 at four days before transplanting (T1) and during transplanting (T2) did not affect flower initiation but reduced the number of flowers than the untreated control.

Table 2. Shoot length, dry weight and root length, dry weight of chilli plants grown in in pots inoculated with (T1, T2, T3, T4) or without BLI008 (T5) at 30 and 75 DAT.

Treatment	30 days after transplanting				75 days after transplanting			
	Shoot[1]		Root[1]		Shoot[1]		Root[1]	
	L*	DW	L	DW	L	DW	L	DW
T1	26.9c	0.83^{cd}	12.5^{b}	0.11^{c}	69.2^{ab}	16.97^{bc}	15.7^{b}	1.37^{b}
T2	25.8^{c}	0.80^{cd}	11.3^{b}	0.09^{c}	65.0^{ab}	13.62^{c}	15.0^{b}	1.35^{b}
T3	21.9^{d}	0.54^{de}	11.0^{b}	0.06^{d}	55.5^{b}	7.85^{d}	13.5^{b}	0.55^{c}
T4	31.1^{b}	1.80^{b}	14.2^{a}	0.23^{b}	75.0^{a}	20.71^{ab}	15.2^{b}	1.54^{b}
T5	14.0^{e}	0.28^{e}	05.2^{c}	0.04^{d}	-	-	-	-
T6	34.9^{a}	2.37^{a}	14.8^{a}	0.42^{a}	79.2^{a}	24.88^{a}	18.5^{a}	2.68^{a}
T7	27.6^{c}	0.95^{c}	12.1^{b}	0.07^{cd}	78.5^{a}	21.11^{ab}	16.0^{b}	1.77^{b}
CV%	8.42	18.18	9.65	14.67	14.34	18.90	10.86	19.44

All plants died in T5 at 75 DAT. Within a row, means followed by the same letter are not significantly different by the DMRT (p=0.05). *L=length (cm), DW=dry weight (g).

Table 3. Increase (+)/decrease (-) in percentages (calculated based on values of untreated control, T7) of shoot and root growth and total biomass of chilli plants in different treatments at 30 and 75 DAT.

Treatment	30DAT			75 DAT		
	Shoot%	Root%	Biomass%	Shoot%	Root%	Biomass%
T1	-2.35	+3.31	-7.84	-11.78	-1.56	-19.84
T2	-6.70	-6.61	-12.75	-17.19	-6.25	-34.57
T3	-26.25	-9.09	-41.18	-29.30	-15.63	-63.29
T4	+12.46	+17.77	+99.02	-4.46	-4.69	-2.75
T5	-59.13	-57.02	-68.63	***	***	***
T6	+24.26	+22.98	+17353	+0.95	+15.63	+20.45
T7	-	-	-	-	-	-

*** No plants in T5 survived up to 75 DAT

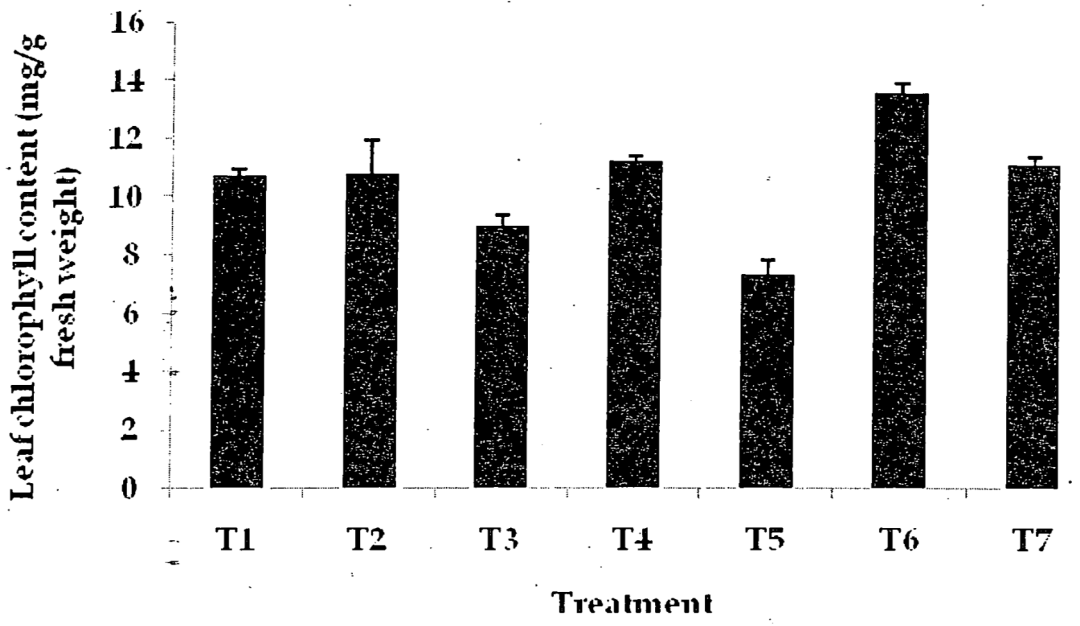

Figure 2 Leaf chlorophyll content at 30 DAT of chilli grown in pots inoculated with (T1, T2, T3, T4) or without BLI008 (T5) at 30 and 75 DAT. Values are the means of eight replicates; vertical bars represent means±SE.

Although T4 registered significantly higher ($p<0.05$) plant growth when compared to the untreated control, the flower initiation and flower production did not show any significant change. The exception was T3 which recorded the lowest number of flowers (4.75) and the time taken for flower initiation was significantly ($p=0.05$) longer (58.75) than untreated control (52).

Table 4. Days taken for flower initiation and number of flowers observed up to 75 DAT (at 2nd destructive sampling) in different treatments.

Treatment	*Days to 1st flowering*	*Number of flowers/plant*
T1	52.0^{b}	16.0^{c}
T2	52.2^{b}	15.0^{c}
T3	58.7^{a}	4.7^{d}
T4	51.7^{b}	21.2^{b}
T5	*	*
T6	48.5^{c}	27.7^{a}
T7	52.0^{b}	23.2^{b}
CV%	3.9	15.9

*No plants survived up to flowering in T5. Wuthin a column, means followed by the same letter are not significantly different by the DMRT ($p=0.05$).

Inoculation with BLI008 significantly reduced ($p<0.05$) *Rhizoctonia* root disease of chilli grown in artificially inoculated soil. Although all treatments showed reduction of the disease, the extent of reduction varied with the time of application. Most probably this is because the antagonistic bacteria mediated disease suppression depends on how they colonize host rhizosphere (Weller, 1988) and on pathogen population near the host (Keinath, 1995). Root disease severity observed in chilli plants revealed that theplants raised in nursery medium colonized with bacteria (T4) performed better than plants treated after transplanting in *R. solani* infested soil (T3). These two cases suggested that pre-treatment with BLI008 did reduce the *Rhizoctonia* disease satisfactorily and more consistent and durable disease protection was observed in plants raised in nursery medium colonized with bacteria. As the nursery medium was sterilized before inoculation, the soil environment did not have any adverse influence on the root colonization or multiplication of the introduced antagonist (Yuan and Crawford, 1995; Chatterton *et al.*, 2004). Abundance of the bacterium in the chilli rhizosphere may result in high concentration of antagonist produced antifungal metabolites (Peypoux *et al.*, 1999; Gdlvez *et al.*, 1993), which positively correlated with the lowest disease severity. Mandeel and Baker (1991) postulated that the root surface has a finite number of infection sites that could be protected by biocontrol agents where profusion of biocontrol agent in the chilli rhizosphere leads to the reduction of infection sites and thereby increase competition for infection sites, ultimately resulting in low root infection.

Though the actual mechanism responsible for disease suppression was not investigated in this study, several possible mechanisms can be suggested to explain the phenomena of disease reduction. Non-pathogenic rhizobacteria-mediated resistance or induced systemic resistance is a powerful mode of action in the biocontrol of both soil borne and aerial plant diseases (Bakker *et al.*, 2003). Several *Bacillus* species have been reported to activate an induced systemic resistance pathway against fungal diseases in plants (Kloepper *et al.*, 2004; Ongena *et al.*, 2005). Harris *et al.* (1994) suggested that the efficacy of bacterial isolates may be improved if they colonize the seedlings before the pathogen, either by prior inoculation or by placement of antagonist inoculum close to the seeds or germinating seedlings. Even though the bacterial inoculation in T1 was done well ahead of transplanting, RDS showed slight differences with T2 while plant growth was similar to that of T2. The reason may be the exposure time of plant roots to the bacterium and pathogen. The bacterium was applied as a cell suspension four days before transplanting in T1 and it probably released some antifungal compounds immediately after introduction to the soil, and reduced the population of the pathogen as suggested by Szczech and Shoda (2004). The low pathogen population of soil in T1 reflects low RDS. Transplanting in pots of T3 was done four days before bacterial application, thus allowing the pathogen to establish a high population

in the rhizosphere. Therefore, the high pathogen density induced more diseases in the roots.

Bacillus licheniformis is capable of producing several antifungal compounds in culture (Peypoux *et al.*, 1999; Gdlvez *et al.*, 1993; Kim *et al.*, 2004). There is much information available on antifungal metabolites produced by bacteria *in-vitro* related to disease suppression *in-vivo* (Heungens and Parke, 2001; Getha and Vikineswary, 2002). Therefore, the antifungal compounds may play a leading role in suppressing the infection in chilli roots. One of the mechanisms used by *Bacillus* species to exert their antagonistic activity against fungal pathogens is parasitism which operates by the degradation of the cell walls of the pathogenic fungi. In this regard, *B. licheniformis* produces the enzyme chitinase that degrades chitin (Trachuk *et al.*, 1996). Due to its ability to degrade chitin, the major structural component of the cell walls of phytopathogenic fungi (Someya *et al.*, 2004), *B. licheniformis* could be considered as an important organism in the biological control of soil borne pathogens.

Plant growth-promoting rhizobacteria (PGPR) inoculation strongly influences the lengths and dry weights of shoots and roots during the early stages of growth (Cakmakci *et al.*, 2006). However, the antagonist-mediated plant growth promotion at the later stages expresses only as an increase of plant biomass. Similar results were reported in some previous studies, showing that PGPR inoculation influence early plant and root development, shoot and root dry weight, and nutrient uptake efficiency of plants (Dobbelaere *et al.*, 2002; Cakmakci *et al.*, 2006). Many plant-associated bacteria have the ability to produce indole-acetic-acid (IAA) that may play a prominent role in plant growth promotion (Patten and Glick, 2002). In general, PGPR may affect the initiation and development of lateral roots (Rolfe *et al.*, 1997), increase root weight, and may have an influence on nutrient uptake potentials (Canbolat *et al.*, 2006). The positive effects of PGPR on plant growth are correlated with changes in root morphology, namely increasing of lateral root and root-hair number and length (Mantelin *et al.*, 2006). Inoculations with the phytohormone producing bacteria produced the highest root weights and total root numbers and encouraged adventitious root formation, but did not affect root length (Cakmakci *et al.*, 2007).

Bacillus licheniformis release gibberellins and auxin-like compounds in culture media (Gutiérrez-Mañero *et al.*, 2001). Gibberellins induce stem and leaf growth, floral induction, and flower and fruit growth (Pharis and King, 1985). Gibberellins are also implicated in the promotion of root growth and root hair abundance (Tanimoto, 2005). Auxins effect root growth, root growth patterns, and increase root-soil surface, resulting in improved nutrient and water absorption potential (Germida and Walley, 1996) influencing plant growth. Cakmakci *et al.* (2007) reported that, *B. licheniformis* RC08 is capable

of dissolving insoluble P in rock phosphate. The increase of nutrient uptake could be expressed as the increase in plant biomass. Therefore, results obtained in this study suggest that BLI008 mediated plant growth promotion is not only limited to the phytohormonal effect but also increase nutrient uptake of the plants.

The positive effects of *B. licheniformis* on chlorophyll concentration were previously reported by Hussain and Hasnain (2009) in cucumber cotyledons. They found that *B. licheniformis* was able to produce cytokinin, a phytohormone which stimulates chloroplast development (Oldroyd, 2007) which correlates with high chlorophyll concentration (Gross, 1991). Furthermore, cytokinins delay the aging of leaves by reducing the degeneration of chlorophyll and enhancing the protein and RNA synthesis (Castelfranco and Beale, 1983). Gibberellins produced by *Azospirillum* spp. and *Bacillus* spp. increased N uptake of wheat roots (Kucey, 1988). The combined effect of cytokinin and gibberellins positively correlates with increased N uptake and chlorophyll synthesis. Therefore, it could be suggested that the stimulation effect of phytohormones on physiological and biochemical processes in chilli plants incited by BLI008 led to increased nutrient uptake and higher photosynthesis, resulting in increased plant growth.

The findings of the present study are in agreement with the effects of root infecting pathogens on chlorophyll concentration, reported on many crop plants (Nogues *et al.*, 2002). There may be several reasons for low chlorophyll concentration in chilli leaves under pathogen attack. Hendry *et al.* (1987) showed that, root and collar diseases induced by pathogens reduce the absorption of important nutrients including N, an essential component of the structure of the chlorophyll molecule. In the present study, even though the potting medium contained additional quantities of N, P, and K, weak translocation towards foliage or the mobilization of nutrients from root to shoot resulted nutrient deficiency which in turn affected the chlorophyll synthesis in infected plants (Sivaprakash *et al.*, 2008). Wilting of the aerial plant parts is one of the obvious symptoms induced by *R. solani* (Sanogo, 2003). Disintegration of host cells in the collar and roots by a pathogen has resulted in sharp decrease of water potential in leaves and lack of water in plant tissue can leads to an increase of oxidative stress, which in turn causes deterioration of chloroplast structure and an associated loss of chlorophyll (Aguirreolea *et al.*, 1995).

The reduced time to flowering and increased number of flowers observed in T6 might be a direct effect of phytohormone gibberellins. This group of phytohormones is involved in several plant development processes and promotes a number of desirable effects including uniform flowering, reduced time of flowering and increase of flower number and size (Jaleel *et al.*, 2009). Production of gibberellins by *B. licheniformis* is well understood

(Gutiérrez-Mañero *et al.*, 2001) and it is possible that gibberellins produced by BLI008 might be responsible for these positive effects. No differences between time to flowering observed in other treatments can be explained by the inhibitory effect of *R. solani* on plant growth, nutrient absorption and leaf chlorophyll content, which can interfere with assimilates in plants, leading to a slowing down of the reproductive process.

CONCLUSIONS

Drenching nursery medium with a suspension of *B. licheniformis* isolate BLI008 (1 x 10^9 CFUs/mL) at seeding protected chilli seedlings from *R. solani*, induced root and collar rots during plant growth. The bacterium is a strong plant growth enhancer and the enhancement is more pronounced at the early stage of growth. Drenching of rhizosphre soil with a suspension of *B. licheniformis* isolate BLI008 (1 x 10^9 CFUs/mL) at transplanting is the appropriate method for growth enhancement.

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