

Bacillus spp. inoculation improves photosystem II efficiency and enhances photosynthesis in pepper plants

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ABSTRACT

Bacillus is one of the main rhizobacteria to have been used as a study model for understanding many processes. However, their impact on photosynthetic metabolism has been poorly studied. The aim of this study was to evaluate the physiological parameters of pepper (*Capsicum chinense* Jacq.) plants inoculated with *Bacillus* spp. strains. Pepper seeds were inoculated with *Bacillus cereus* (K46 strain) and *Bacillus* spp. (M9 strain; a mixture of *B. subtilis* and *B. amyloliquefaciens*), chlorophyll fluorescence and gas exchange were evaluated. The ANOVA ($P \leq 0.05$) showed that the maximum photochemical quantum yield of photosystem II (PSII) (F_v/F_m) in plants inoculated with the M9 strain (0.784) increased with respect to other treatments (K46: 0.744 and Control: 0.739). Inoculated plants with M9 and K46 strains exhibited an increase of both photochemical quenching (qP) (by 27% and 24%, respectively) and CO₂ assimilation rate (photosynthesis) (by 20% and 16%, respectively), when compared with non-inoculated plants. Furthermore, plants inoculated with M9 and K46 showed decreased transpiration (61% and 57%, respectively) with respect to controls. Likewise, both electron transport rate of PSII (ETR) and PSII operating efficiency (Φ_{PSII}) increased in inoculated plants. However, only plants inoculated with the M9 strain showed enhancements on all growth characteristics. Our results therefore show that inoculating plants with M9 strain positively influenced the performance of the photosynthetic mechanism in pepper plants to increase chlorophyll fluorescence and gas exchange parameters. Promotion of photosynthetic capacity in pepper was due to increased ETR in the thylakoid membranes, which was promoted by the bacteria. M9 strain could even be used in sustainable agriculture programs.

Key words: *Capsicum chinense*, chlorophyll fluorescence, CO₂ assimilation rate, plant growth promoting rhizobacteria.

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INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a diverse group of bacteria that can be found in the rhizosphere, on root surfaces and in association with roots (Ahemad and Kibret, 2014; Lucas et al., 2014). Rhizobacteria are proficient at colonizing the root surface, surviving, multiplying and competing with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, which can improve plant fitness by different mechanisms (García-Cristobal et al., 2015). Directly, the presence of rhizobacteria can cause modifications to plant metabolism. Examples include N fixation, phosphate solubilization, Fe sequestration, and cytokinin, gibberellin, indoleacetic acid and ethylene production (Lucas et al., 2014). Indirectly, the presence of rhizobacteria promotes mechanisms that do not involve plant metabolism. Examples include antibiotics; lytic enzymes, such as chitinases, cellulases, 1,3-glucanases, proteases and lipases; siderophore production; competition between pathogens and non-pathogens; induced systemic resistance; and modulation of environmental stress effects (Ahemad and Kibret, 2014). Although the mechanisms used by rhizobacteria are well known, their impact on photosynthetic metabolism remains unclear (Shi et al., 2010; Stefan et al., 2013a; Palacios et al., 2014).

Some studies have reported that PGPR affect the physiology of plants to attenuate to some degree the stressful effects of drought, salt, UV, and a combination of high CO₂ content and low atmospheric pressure (Burlak et al., 2013; Stefan et al., 2013b; García-Cristobal et al., 2015). Actually, some studies have shown that bacterial capacity to reduce reactive oxygen species (ROS) levels in drought stressed plants and were also correlated with an increase in photosynthesis (Armada et al., 2014). Thus, Shi et al. (2010) showed that endophytic bacteria species increased the photosynthetic capacity and total chlorophyll content of sugar beet, leading to a consequent increased carbohydrate synthesis, these increases were promoted by phytohormones (indole-3-acetic acid, IAA) which were produced by the bacteria. The main parameters used to sensitively and objectively determine the ability of the strains to affect the photosynthetic apparatus in plants are minimum fluorescence (F_0), maximum photochemical quantum yield of photosystem II (PSII) (F_v/F_m), PSII operating efficiency (Φ_{PSII}), non-photochemical quenching (NPQ), and electron transport rate (ETR) (Lucas et al., 2014; García-Cristobal et al., 2015). *Bacillus* is one of the main rhizobacteria that has been used as a study model for understanding many processes, because it has been found to possess a wide genetic diversity adapted to different conditions and numerous properties of

interest applied in industry, microbiology and agriculture (Niazi et al., 2014). Inoculation with *B. subtilis* increase photosynthesis in Arabidopsis through the modulation of plant endogenous sugar/abscisic acid (ABA) signaling, with a regulatory role for plant symbionts in photosynthesis (Zhang et al., 2008). However, Freitas et al. (2015) showed that iron accumulation in cassava was accompanied by an increase in the photosynthetic rate and biomass accumulation and may be linked with greater chlorophyll levels at this location. In this way, *B. subtilis* has been shown to increase the abundance of transcripts involved in iron uptake and transport as well as induce rhizosphere acidification that directly mobilizes soluble minerals (Zhang et al., 2009).

Furthermore, peppers (*Capsicum* spp.) are an economically important genus of the Solanaceae family and pepper plants are grown around the world because of their adaptation to different agro-climatic regions (Kraft et al., 2014). In particular, Habanero pepper (*Capsicum chinense* Jacq.) grows in wide temperature range, and has therefore been used in studies of plant stress (Garraña-Hernández et al., 2014; Valle-Gough et al., 2015).

Although studies have been performed on the effect of *Bacillus* in *C. chinense* (Kanchana et al., 2014; Gan et al., 2015), there are no previous studies on the effect of *Bacillus* spp. on the photosynthetic mechanisms of *Capsicum* spp. Therefore, two specific questions are addressed here: What are the physiological responses of *C. chinense* plants to inoculation with *Bacillus* spp. strains? Does photosynthesis increase in response to plant-rhizobacteria symbiosis? In order to contribute to the understanding of photosynthetic mechanisms during interactions between plants and rhizobacteria, the aim of this study was to evaluate the physiological parameters of *C. chinense* plants inoculated with *Bacillus* spp. strains.

MATERIALS AND METHODS

Bacillus spp. strains and plant material

Bacillus cereus (K46 strain) and *Bacillus* spp. (M9 strain; a mixture of *B. subtilis* and *B. amyloliquefaciens*) were isolated from soils in the state of Yucatán, Mexico. Strains were grown in 75 mL of nutrient broth under constant stirring at 200 rpm for 96 h at 30 °C. Subsequently, the cultures were centrifuged at 3500 rpm for 15 min in a centrifuge (C-600 SOLBAT) at 29 °C to obtain the cells, which were washed twice with sterile saline 0.8% and dissolved in 2 mL saline for counting in a Neubauer chamber. The cell concentration was adjusted to 10^8 cells mL^{-1} in 10 mL saline 8%. Twenty seeds per tube were placed in a centrifuge for 1 h at 160 rpm.

The H241 genotype of Habanero pepper (*Capsicum chinense* Jacq.) was used, and seeds were disinfected with sodium hypochlorite 2% and washed three times with sterile distilled water. Sterile seeds were sown in germination trays with 200 cavities filled with sterile peat moss substrate

moistened to field capacity with distilled water according to the experimental design. All trays were placed in a growth room at 20 ± 2 °C. They were watered every second day and leaf fertilized (UltraFol, Biochem, Constituyentes, México) weekly at a dose of 1 g L^{-1} distilled water. After 18 d germination, seedlings were transferred to 500 mL Styrofoam cups filled with sterile peat moss substrate. The seedlings were kept in a growth room at 20 ± 2 °C and were watered and fertilized every second day as mentioned above. Ninety seedlings were randomly selected 60 d after seed sowing (DASS) and placed in a greenhouse.

Chlorophyll fluorescence and gas exchange analyses

The chlorophyll (Chl) fluorescence parameters were measured *in vivo* with a portable pulse amplitude modulation fluorometer (PAM Walz, Effeltrich, Germany). Measurements were performed 145 d after sowing at pre-dawn (05:00 h), on the third leaf from the shoot apex. The parameter F_v/F_m represents the maximum photochemical quantum yield of PSII when reaction centers are open after dark adaptation, where F_v is maximum variable fluorescence (defined as $F_m - F_0$), F_0 is minimum Chl fluorescence yield in the dark-adapted state, and F_m is maximum Chl fluorescence yield in the dark-adapted state. To determine the F_0 on excitation of leaf with a weak measuring beam. The F_m was determined with a 0.6 s saturating pulse. After, an actinic light of $420 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was switched on and saturating pulses ($8000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) were applied at 10 min intervals to determine the maximum fluorescence intensity in the light-adapted state (F_m'), minimum fluorescence intensity in the light-adapted state (F_0') and steady state fluorescence intensity in the light-adapted state (F_s) (Calatayud et al., 2002). The potential activity of PSII (F_v/F_0) was obtained according to Li et al. (2007). Both electron transport rate ($\text{ETR} = \Phi_{\text{PSII}} \times \text{PAR} \times 0.5 \times \text{leaf absorptivity coefficient}$) and PSII operating efficiency ($\Phi_{\text{PSII}} = (F_m' - F_s')/F_m'$) were obtained through the application of a series of nine saturation pulses under increasing actinic irradiance (PAR) from 0 to $1500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The photochemical quenching ($\text{qP} = (F_m' - F_s)/(F_m' - F_0')$) and non-photochemical quenching ($\text{NPQ} = F_m/F_m' - 1$) were calculated according to Kalaji et al. (2014).

CO_2 assimilation rate (A_N), stomatal conductance (g_s) and transpiration (E) were measured using an infrared gas analyzer (IRGA; LICOR, LI-6400, Lincoln, Nebraska, USA). Water use efficiency (WUE) was calculated as A_N/E (Garraña-Hernández et al., 2014). Measurements were performed in the same leaves used in chlorophyll fluorescence measurement. The greenhouse conditions were 28 °C, photosynthetic active radiation $1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and 65% RH.

Total chlorophyll concentration was measured by SPAD-502 portable chlorophyll meter (Minolta, Osaka, Japan) in the same leaves used previously.

Experimental design

Thirty plants per treatment were used in a completely random design. The results were analyzed with one-way ANOVA and means were compared using Tukey's test at $P \leq 0.05$ (Statistica Six, StatSoft, Tulsa, Oklahoma, USA).

RESULTS

Growth parameters and chlorophyll fluorescence parameters

All treatments were significantly similar for plant height, but stem diameter, fresh weight and root volume of plants inoculated with *Bacillus* sp. M9 increased with respect to control plants by 22%, 34% and 58%, respectively (Table 1).

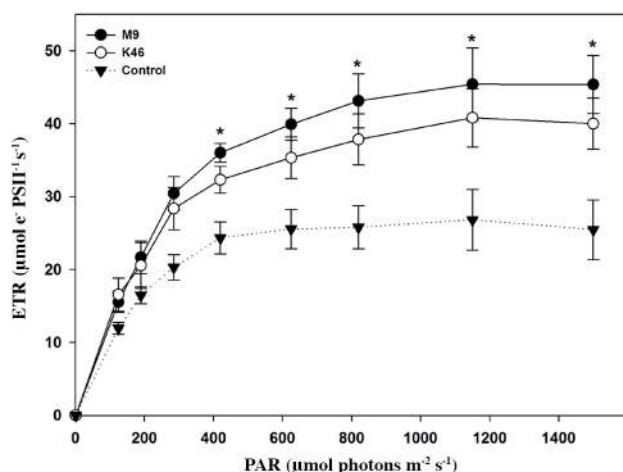
The electron transport rate (ETR) of PSII increased in leaves of plants inoculated with M9 and K46 strains. ANOVA showed significant differences between treatments at above $420 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. At $1150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ all treatments reached the maximum ETR (M9: 45.4; K46: 40.8 and control: 26.8) (Figure 1). PSII operating efficiency (Φ_{PSII}) was higher in inoculated plants than control plants from lowest to highest photosynthetic photon flux density (Figure 2). The maximum photochemical quantum yield of PSII (F_v/F_m)

Table 1. Growth parameters of Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).

Treatment	Plant height	Stem diameter	Fresh weight	Root volume
	cm	mm	g	mL
M9	180 ± 2.3	$27 \pm 1.7a$	$1657 \pm 133a$	$516 \pm 96a$
K46	165 ± 3.0	$20 \pm 1.1ab$	$1051 \pm 64b$	$483 \pm 92ab$
Control	176 ± 7.2	$21 \pm 2.1b$	$1093 \pm 106b$	$216 \pm 60b$

Data are means \pm SE. Different letters in the columns represent significant differences according to Tukey test ($\alpha = 0.05$).

Figure 1. Response curves of electron transport rate (ETR) to photosynthetic photon flux density in Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).

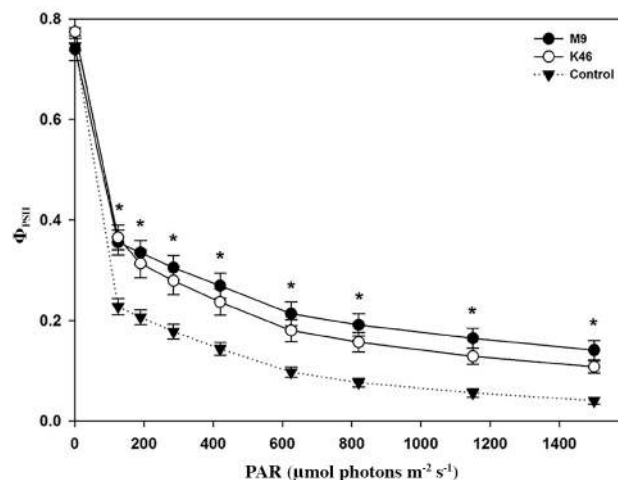


Data are means \pm SE.

NS: nonsignificant, PSII: photosystem II.

*Significant between treatments at 0.05 probability level.

Figure 2. Photosystem II (PSII) operating efficiency (Φ_{PSII}) to photosynthetic photon flux density in Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).



Data are means \pm SE. NS: nonsignificant. *Significant between treatments at 0.05 probability level.

of plants inoculated with M9 strain (0.784) increased with respect to plants inoculated with K46 (0.744) and control plants (0.739) (Figure 3A). On the other hand, the potential activity of PSII (F_v/F_0) showed a significantly similar trend to F_v/F_m . Values for plants inoculated with M9 strain (3.6) were 20% higher than for non-inoculated plants (2.9) (Figure 3B). Plants inoculated with M9 and K46 strains showed an increase in qP with respect to control plants (27% and 24%, respectively) (Figure 3C). Likewise, plants with M9 strain (1.18) increased the NPQ 26% with respect to control plants (0.88) (Figure 3D).

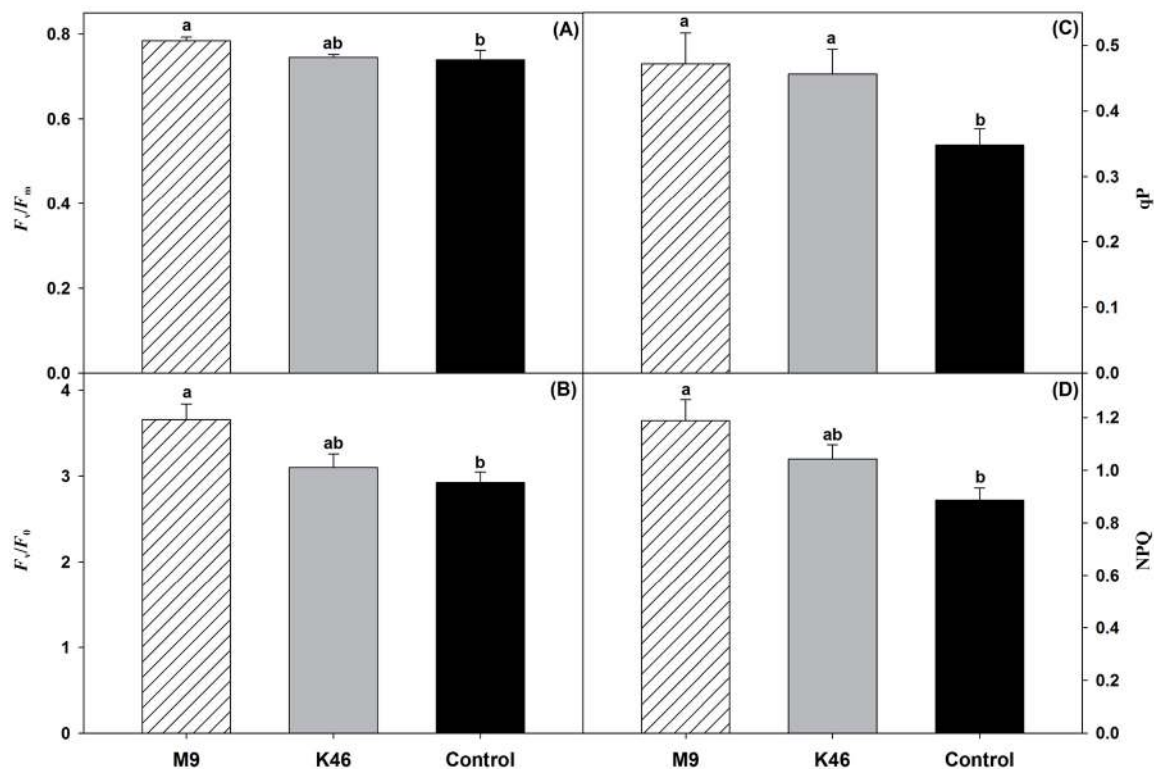
Gas exchange analyses

Inoculation with M9 and K46 increased the CO_2 assimilation rate (photosynthesis) in Habanero pepper plants by 20% and 16%, respectively, compared to non-inoculated plants (Figure 4A). Plants inoculated with M9 and K46 also showed decreases in both stomatal conductance (56% and 45% respectively) (Figure 4B) and transpiration (61% and 57% respectively) (Figure 4C). However, the two strains improved WUE by 68% and 66% respectively (Figure 4D). On other hand, plants inoculated with the M9 increased SPAD values with respect to non-inoculated plants (7%), but plants inoculated with the K46 had lower values than control plants (Figure 5).

DISCUSSION

Plant growth promoting rhizobacteria (PGPR) are known to favor plant growth via biological N fixation (Ashok et al., 2015), regulation of the synthesis of plant growth-regulators (Ahemad and Kibret, 2014) and solubilization of soil-insoluble compounds such as calcium di- and tri-phosphates and other minerals (Marschner, 2007). According to our results, inoculating plants with *Bacillus* spp. strains

Figure 3. A) Maximum photochemical quantum yield of photosystem II (PSII) (F_v/F_m), B) potential activity of PSII (F_v/F_0), C) photochemical quenching (qP) and D) non-photochemical quenching (NPQ) in Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).



Actinic light intensity: $420 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Data are means \pm SE. Different letters represent significant differences between treatments according to Tukey test ($\alpha = 0.05$).

modified the physiological mechanisms with respect to control plants (not inoculated).

The electron transport rate (ETR) of PSII increased in leaves of plants inoculated with *Bacillus* spp. strains. According to Li et al. (2007), the ETR of PSII is inhibited by stress. However, the plants in this study were not subjected to stressful conditions. Therefore, the increase in ETR values is a consequence of the positive effect of PGPR. According to Melis (1999), an increase in the electron transport rate of PSII suggests that the quinone acceptor (Q_A) is highly oxidized and its excitation energy is utilized in electron transport, thereby avoiding photodamage. Inoculating plants therefore has the potential to increase photosynthesis rates and plant growth.

Additionally, it is observed that PSII operating efficiency (Φ_{PSII}) increased in inoculated plants. This parameter measures the proportion of light absorbed by chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson, 2000). Habanero pepper plants inoculated with *Bacillus* spp. strains therefore showed an increase in overall photosynthetic capacity *in vivo*. Zhang et al. (2007) showed that certain PGPR elevate photosynthesis in *Arabidopsis* through the modulation of endogenous sugar/abscisic acid (ABA) signaling and establish a regulatory role for soil symbionts in plant energy acquisition. Some *Bacillus* are known to possess volatile organic compounds that function as modulators, sensing primary and secondary metabolism,

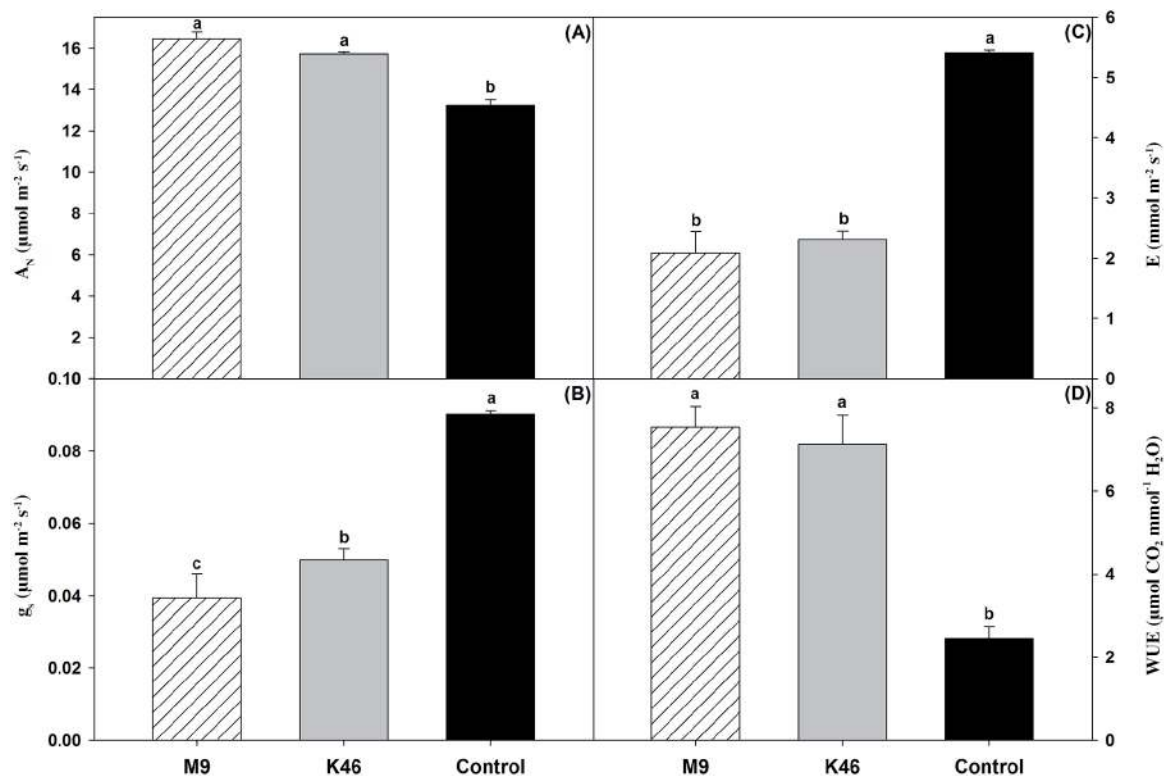
stress responses and hormone regulation in plants (Zhang et al., 2007).

Moreover, the maximum photochemical quantum yield of PSII (F_v/F_m) increased in inoculated plants. According to Baker (2008), F_v/F_m is the maximum efficiency at which light absorbed by PSII is used for reduction of Q_A , and is used as a sensitive indicator of plant photosynthetic performance. An F_v/F_m value of around 0.83 is an optimal measurement for most plant species (Maxwell and Johnson, 2000). This suggests that the M9 strain promoted a healthy PSII in inoculated plants, and other authors obtained similar results (Gururani et al., 2012), with increased F_v/F_m values when using PGPR.

On other hand, the potential activity of PSII (F_v/F_0) showed a similar trend to F_v/F_m . Li et al. (2007) found a decrease of 21.7% in F_v/F_0 after 48 h water stress in *Medicago sativa*. Yang et al. (2008) mentioned that PGPR also elicit so-called 'induced systemic tolerance' to salt and drought. Our results therefore showed that inoculation with PGPR could prepare the plant for future abiotic stress situations.

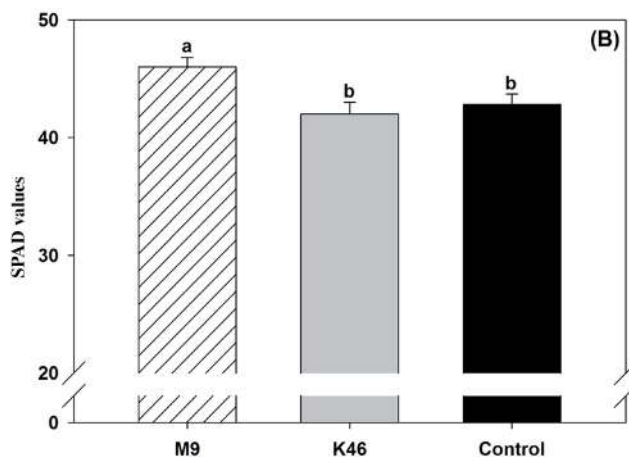
Plants inoculated with M9 and K46 had an increase in qP with respect to control plants; qP denotes the proportion of excitons captured by open traps and being converted to chemical energy in the PSII reaction center (Krause and Weis, 1991).

Figure 4. A) CO₂ Assimilation rate (A_N), B) stomatal conductance (g_s), C) transpiration (E), and D) water use efficiency (WUE) of Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).



Data are means ± SE. Different letters represent significant differences between treatments according to Tukey test ($\alpha = 0.05$).

Figure 5. SPAD values of Habanero pepper plants inoculated with *Bacillus* spp. (M9 and K46) and not inoculated (control).



Data are means ± SE. Different letters represent significant differences between treatments according to Tukey test ($\alpha = 0.05$).

Likewise, NPQ was higher in plants inoculated. This study therefore showed that inoculated plants had a better way of getting rid of excess light energy that had already been absorbed, because of the non-photochemical mechanisms that quench singlet-excited chlorophylls (Chl) and harmlessly dissipate excess excitation energy as heat (Müller et al., 2001). Both qP and NPQ help to minimize production of O₂ in the PSII antenna (Müller et al., 2001).

On the other hand, our results therefore show that plants inoculated with *Bacillus* strains modified gas exchange to enhance CO₂ assimilation (photosynthesis) and reduce water release by leaves. Water use efficiency in inoculated plants was therefore better, assimilating more CO₂ molecules per H₂O molecule released by stomata. Some authors (Vivas et al., 2003; Vardharajula et al., 2011; Armada et al., 2015) mention that plants inoculated with PGPR developed drought tolerance. According to Kumar et al. (2015), PGPR influence the physiology of the whole plant and play a key role in plant growth and the yield of various crops. Plants are known to release part of their photosynthetic output in the form of root exudates that attract and maintain bacterial colonies in the soil, promoting mutually beneficial effects, including the enhancement of plant abiotic stress tolerance by PGPR (Liu and Zhang, 2015).

Additionally plants inoculated with M9 increased SPAD values with respect to non-inoculated plants. Marulanda-Aguirre et al. (2008) observed decreased photosynthetic pigments (chlorophyll and carotenoids) in *Lactuca sativa* plants inoculated with *Bacillus megaterium*, but photosynthetic pigments increased when *B. megaterium* was combined with an arbuscular mycorrhizal fungus. The effect of PGPR on photosynthetic pigments may therefore depend on the strain used for inoculation.

Moreover, it was observed that the inoculation with the *Bacillus* spp. M9, increased plant growth. Inoculation

with PGPR was found to improve plant growth in previous studies on pepper plants, Kang et al. (2007) used *Pseudomonas rhodesiae* and *Pantoea ananatis* and promoted significant growth of peppers, enhancing their root fresh weight by 73.9% and 41.5%, respectively. Datta et al. (2011) found that the inoculation of pepper plants with strains of *Bacillus* spp. and *Streptomyces* spp. increased growth characteristics such as total number of fruits, fruit weight and yield. Luna et al. (2013) evaluated pepper plants inoculated with *Bacillus* spp. strains and observed an increase in biomass. There are different mechanisms explaining how PGPR favor plant growth: Biological N fixation (Ashok et al., 2015); regulation of the synthesis of plant growth-regulators, which can promote root growth and proliferation of root hairs, enhancing the uptake of water and minerals from the soil (Ahemad and Kibret, 2014); and a decrease in ethylene content of the developing or stressed plants, inducing elongation of the root system (Vessey, 2003).

Although some physiological parameters increased with K46 strain, plant growth parameters were similar from those of controls. According with Ahemad and Kibret (2014) the plant-beneficial symbiotic interactions are influenced by root exudates, composition of these exudates is dependent upon the physiological status and species of plants and microorganisms, probably K46 strain has not an excellent synergy with pepper plants, and its increase in photosynthesis was not enough to increase biomass. However, all plant growth parameters improved in pepper plants inoculated with M9 strain with respect to control plants. The microorganisms can promote plant growth by regulating nutritional and hormonal balance, producing plant growth regulators and solubilizing nutrients (Nadeem et al., 2014). According to Patten and Glick (1996) the microorganisms isolated from rhizosphere synthesize auxins (indole-3-acetic acid/indole acetic acid/IAA). The IAA affects plant cell division, pigment formation and photosynthesis by changing the plant auxin pool (Ahemad and Kibret, 2014). Moreover, CO₂ resulting from respiration of bacteria can help to increase photosynthesis. Hibberd and Quick (2002) reported that CO₂ produced in roots can be transported to the shoot; stem cells in tobacco are supplied with C for photosynthesis from the vascular system and not from stomata. In this way, Rozpadek et al. (2015) suggest that upon endophyte colonization, host plant undergoes changes in its photosynthetic apparatus, leading to increased light harvesting and photosynthesis efficiency.

Here, M9 strain improved chlorophyll fluorescence parameters and total chlorophyll concentration, suggesting more reaction centers and higher light harvesting; thus, the quinone acceptor (Q_a) is highly oxidized and its excitation energy is utilized in electron transport, leading higher ATP and NADPH production, employed for C assimilation in the Calvin cycle, and improving plant growth.

CONCLUSION

Based on our results, we concluded that M9 strain (*Bacillus* spp.) used in this study demonstrated a striking influence on all parameters evaluated. Both strains (M9 and K46) improved the efficiency of photosystem II (PSII) to increase both the electron transport rate (ETR) and PSII operating efficiency (Φ_{PSII}) of inoculated plants. However, M9 increased photochemical quenching (qP), non-photochemical quenching (NPQ), maximum photochemical quantum yield (fluorescence) and potential activity of PSII. In addition, plants inoculated with M9 and K46 showed remarkably improved gas exchange, increasing both the net assimilation rate (photosynthesis) and water use efficiency (WUE), and decreasing transpiration rate. Inoculation with *Bacillus* spp. (M9) and *B. cereus* (K46) therefore positively influenced the performance of the photosynthetic mechanism in Habanero pepper plants. However, plants inoculated with the M9 strain showed enhancements on all growth characteristics. M9 strain could be used in sustainable agriculture programs.

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