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Use of plant growth promoting Rhizobacteria (PGPR) and mycorrhizae to improve the growth and nutrient utilization of common bean in a soil infected with white rot fungi

Ibrahim Mohamed[a,b,c,*], Khaled E. Eid[d], Mohamed H.H. Abbas[b], Ahmed A. Salem[e], Nevin Ahmed[f], Maha Ali[b], Ghulam Mustafa Shah[g], Chen Fang[a,c,*]

[a] Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Science, Wuhan 430074, China
[b] Soil and Water Sciences Department, Faculty of Agriculture, Benha University, Egypt
[c] China Program of International Plant Nutrition Institute, Wuhan 430074, China
[d] Plant Pathology Department, Faculty of Agriculture, Benha University, Egypt
[e] Agricultural Microbiology Department, Faculty of Agriculture, Benha University, Egypt
[f] Benha University, Benha, Al-Qalyubia Governorate, Egypt
[g] Department of Environmental Sciences, COMSATS University Islamabad, Vehari-campus, Vehari 61100, Pakistan

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ABSTRACT

Extensive use of fertilizers and pesticides led to dangerous ecological effects and therefore the biological approaches have been widely recommended to prevent further deterioration for the environment. The current study was conducted to explore the potentiality of using single or combined inoculations by mycorrhizae, *Bacillus subtilis* and *Pseudomonas fluorescence* for controlling the infection of common bean plants with *Sclerotium rolfsii* on one hand and as bio-fertilizers for improving plants nutritional status on the other hand. The soil of study was mildly infected with *S. rolfsii* and contained high total-P content. Thus, minimal P inputs were added to the inoculated soil in the form of rock phosphate. Activities of plant defense enzymes i.e. chitinase, peroxidase and polyphenol oxidase were determined under the greenhouse conditions and the results obtained herein indicated that activities of such enzymes increased significantly owing to bio-agent inoculations. In this concern, combined treatments resulted in further significant increases over the single ones. A field study was then conducted for two successive years and the results reveal that single inoculations increased straw and green pod yields as well as the uptake of P and Fe by plants as compared with the non-inoculated treatment. Combined inoculants recorded further significant increases in these parameters even when compared with the fungicide treated plants. Generally, straw and pod yields obtained from the second growing season were significantly higher than those attained in the first growing one. Our study confirms the success of the used bio-treatments in minimizing soil pollution through fertilizer and/or pesticide inputs.

1. Introduction

Extensive use of fertilizers and pesticides during the last couple of decades led to successful intensive agricultural practices (Chowdhury et al., 2008). However, some dangerous ecological effects have been detected with the continuous use of these chemical fertilizers, beside of the inhibition in the proliferation of beneficial soil microorganisms and their bio-transformations which is associated with the extensive use of chemical pesticides (Hussain et al., 2009). Alternatively, introducing certain biological agents might be effective, to some extent, as bio-fertilizers and, at the same time, offer different mechanisms for controlling plant disease rather than chemical pesticides (Fravel, 2005; Mehta et al., 2014; Luo et al., 2018).

Plant roots release carbon compounds of low molecular weights which promote the growth of beneficial bacteria in soil rhizosphere (Tarkka et al., 2008). These beneficial bacteria can act as the source and sink of minerals in soil, and/or stimulate plant hormones thus increase the plant growth; beside of the ability of some bacterial types to inhibit the growth of soil-borne pathogen (Hampp and Tarkka, 2009). In this concern, *Sclerotium rolfsii* is a soil-borne pathogen which attacks various

* Corresponding authors at: Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Science, Wuhan 430074, China.
E-mail addresses: Ibrahim.ali@fagr.bu.edu.eg (I. Mohamed), fchen@ipni.ac.cn (C. Fang).

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crops (Adandonon et al., 2006) causing enormous economic losses in agriculture (Faria et al., 2009; Eid and Abbas, 2014). It is difficult to control white rot fungi due to the formation of sclerotia resistant structures (Faria et al., 2009), which may exist in soils under unfavorable conditions for years (Zhong et al., 2016; Tarafdar Avijit et al., 2018). However, some bacterial types can inhibit the growth of plant pathogens (Hampp and Tarkka, 2009). It is, therefore, thought that the ascendancy of soil-borne pathogens might result from the microbial disorders in the rhizosphere of infected plants. Thus, inoculating plants with appropriate species of symbiotic microorganisms might be suitable to maximize the efficiencies of the host plants to use soil nutrients and, at the same time, arise above the ascendancy of this pathogen in soil, consequently enhance plant growth and the edible yield productivity.

Plant growth promoting Rhizobacteria (PGPR) are micro-organisms that exist in the rhizosphere and are "involved directly or indirectly in promoting plant growth and development" (Prasad et al., 2019) i.e. *Bacillus* and *Pseudomonas* sp. because of their low cost and simple method of application, thus PGPR are recommended in sustainable agriculture to improve the nutritional status of the grown plants on one hand, and suppress soil-borne pathogens on the other hand (Verma et al., 2018). *Pseudomonas fluorescens* and *Bacillus subtilis* can be used successfully as biological agents for *S. rolfsii* (Mundhe et al., 2009), in addition to the mycorrhizael inoculation for plant seeds which can effectively control stem rot infection caused by *S. rolfsii* (Ozgonen et al., 2010). Moreover, micorrhizael external hyphaes can improve the nutrient supply to the grown plants especially the immobile ones; besides, they increase the P- depletion zone (Lambers et al., 2018). In case of *B. subtilis* and *P. fluorescens*, they are plant growth promoting bacteria (Sivasakthi et al., 2013) which can increase the solubility and availability of P in soil (Zaidi et al., 2017). Besides, they induce high-affinity Pi transporters in plant roots and hence increase P uptake by grown plants (Belgaroui et al., 2016; Ian et al., 2017). Moreover, the potentiality of these two microbes to remove high concentrations of nutritive metals from soils is also thought to be high thus limits their availability to the pathogen (Zaidi et al., 2017) i.e. *P. florescence* removes high concentrations of Fe from the medium; thus, limits its availability to the pathogen fungi (Beneduzi et al., 2012); whereas, *B. sitlus* chelates soil-Fe and significantly increase its availability for plants (Freitas et al., 2015). Probably, introducing bioagents to control plant pathogens, which act also as biofertilizers, might improve the nutritional status of the infested plants and consequently stimulate the induced resistance of plants towards the soil-borne pathogens. Thus, mineral fertilizers are recommended to investigate, to what extent, the dual efficiency of the used bioagents can successfully control the existing plant pathogens. It is worthy to mention that plant co-inoculation with either *P. Fluorescens* or *B. subtilis* can further promote the colonization of plant roots by arbuscular mycorrhizal fungi (AMF) (Priyadharsini and Muthukumar, 2016). Therefore, the current study aims at evaluating the effects of inoculating common bean seeds with mycorrhizae, *B. subtilis* and *P. Fluorescens* either solely or in different combinations under minimal inputs of fertilizers on controlling the plant pathogen and, at the same time, improving the nutritional status of the grown plants; thus, maintaining land sustainability.

2. Materials and methods

2.1. Materials of study

To explore the objectives as outlined above, a glasshouse experiment and 2-years experiment in the field were conducted at the Experimental Farm of the Horticulture Department, Faculty of Agriculture, Benha University, Egypt. The soil of study was sampled prior to the field study, air-dried, crushed and sieved to pass through a 2 mm sieve, and then analyzed for physical and chemical properties according to the standard methods. Soil texture was classified as clay (USDA) with coarse sand, fine sand, silt, clay of 5.7%, 14.3%, 31%, and 49%, respectively. Further, soil analysis revealed the following characteristics: pH 7.64, EC 1.63 dS m^{-1}, OM 13.8 g kg^{-1}, total P 38.34 g kg^{-1}, potentially plant-available P (e.g, Olsen-P)51.32 and total K of 5083 mg kg^{-1}.

Bioagents i.e. *Bacillus subtilis* (DSM1088) and *Pseudomonas fluorescens* (ATCC13525) were obtained from the Microbial Research Center (The Cairo MIRCEN, Egypt Microbial Culture Collection (EMCC)). *Rhizobium leguminsarium* ICARDA441 was kindly provided by Biofertilizers Production Unit, Agricultural Microbiology Department, Soil Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza, Egypt. *Mycorrhiza* (*Glomus* spp) was supplied by the Agricultural Research Station, Agricultural Research Center (ARG, Egypt). Seeds of common beans (*Phaseols vulgaris* cv. Bronco) were obtained from the Department of Vegetable and Crops Research, Agricultural Research Center (ARC), Giza, Egypt.

2.2. Identification of the white rot pathogen

Bean plants (*Phaseols vulgaris* L.) grown on the studied area showed the symptoms of white moldy layer with small, smooth and brown sclerotia found at the portions in contact with soil. These symptoms were identified according to Schwartz et al. (2005) as *Sclrotium rolfsii* infection. Further morphological identifications of *S. rolfsii* were carried out under the light microscope of the Department of Fungal Taxonomy, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza-Egypt. This soil was thought to be mildly infected with *S. rolfsii.*

2.3. Methods of study

To attain the purposes of the study, *P. flourescens* and *B. subtilis* were grown in conical flasks (250 mL), each containing 100 mL of nutrient broth medium and then incubated on a rotary shaker with an agitation rate of 200 rpm for 72 h at 28 ± 2 °C. Afterwards, the suspensions of the bacteria were adjusted to 2.2×10^8 cfu mL^{-1}. *S. rolfsii* was grown for 15 days at 28 ± 2 °C in 500 mL glass bottles containing autoclaved sand medium consisting of 25% clean sand, 75% sorghum grains and sufficient amount of distilled water to cover the mixture.

2.4. Propagation of AMF cultures

Common bean seeds were surface-sterilized with ethanol for 2 min and then NaClO solution for 10 min (0.75% Cl) as outlined by Varma et al. (1999); thereafter, washed several times with sterile water and soaked in deionized water for 5 min at 60 °C to minimize the presence of *Fusarium*. Seeds were then germinated on water-agar plates (0.8% Bacto Agar, Difco) and incubated in dark for 3 days at 25 °C (Varma et al., 1999). Soil-sand (2:1 w/w) mix substrate portions were sterilized in an autoclave at 15 lb for half an hour to kill the indigenous propagules and then uniformly packed in 2 kg sterilized plastic pots (25 cm diameter × 18 cm depth). Five bean seeds were placed in each pot together with 50 g in vitro-produced spores, soil and colonized roots. Two months later, the whole roots were removed gently from soil and washed several times with sterilized distilled water to remove the adhered soil particles. Heavily colonized roots were selected under a light microscope at × 200 and × 400 magnification, air dried to be used later as inoculums.

2.5. The greenhouse experiment

This experiment was a preliminary one conducted under the greenhouse conditions to investigate the effectiveness of inoculating common bean plants with bacillus, pseudomonas and AMF (*Glomus* spp) either solely or in combinations on the growth performance of common beans grown on a soil infected with *Sclerotium rolfsii*. For this purpose, plastic pots (25 cm in diameter) were sterilized by dipping in 5% formalin solution for 5 min, washed with tap water and then left to

get rid of the remained formalin for 2 weeks to ensure complete evaporation of formalin. The sterilized pots were packed with 2 kg of sand: soil (1:3). The inoculum of *S. rolfsii* was mixed thoroughly with the soil at the rate of 30 g kg^{-1} and then all pots were regularly watered with tape water for a week to maintain soil moisture at the field capacity. Afterwards, AMF inoculum (vitro-produced spores, soil and colonized roots) was applied to the infested soil at a rate of 30 g kg^{-1}. Bean seeds were coated by the a suspensions of *B. subtilis* or *P. flourescense* at a rate of 100 mL cell suspension kg^{-1} seeds (contains about 2.2×10^8cfu mL^{-1}) for 5–10 min in presence of 10% Arabic gum solution as an adhesive agent, and then dried in dark at room temperature. Additional suspension was added through soil drench after the emergence of two leaves to increase the rate of colonization of the bacterial cells. The fungicide treatment took place through dressing seeds with a film of Vitavax- 200 at a rate of 3 g kg^{-1} seed. The experiment was conducted in a complete randomized design with five replicates. The treatments involved: single inoculation with *Bacillus subtilis* (T1), combined inoculation with *Bacillus subtilis* and mycorrhiza AMF (T2), single inoculation with *Pseudomonas fluorescens* (T3), combined inoculation with *Pseudomonas fluorescens* and AMF (T4), single inoculation with AMF (T5), Vitavax- 200 fungicide (T6) and no inoculation (control) treatment (T7). Five seeds of common beans were planted in each pot. All pots received 40 mg N kg^{-1} in the form of ammonium sulfate fertilizer (205 g N kg^{-1}), 6 mg P kg^{-1} in the form of rock phosphate and 40 mg K kg^{-1} in the form of potassium sulfate (200 kg K kg^{-1}). Soil surfaces in all pots were covered with acid washed sand to keep the soil warm. At the 15th, 30th and 60th days of planting (DAP), pre- and post-emergence damping-off percentages as well as the percentage of survived plants were calculated according to the following equations:

$$\text{Pre} - \text{emergence } (\%) = \frac{\text{number of non} - \text{germinated seeds}}{\text{number of sown seeds}} \times 100 \quad (1)$$

$$\text{Post} - \text{emergence } (\%) = \frac{\text{number of rotted seeds}}{\text{number of sown seeds}} \times 100 \quad (2)$$

$$\text{Plant survival } (\%) = \frac{\text{number of survived plants}}{\text{number of sown seeds}} \times 100 \quad (3)$$

Samples of bean leaves (equivalent to 5 g each) were collected six weeks after planting from each treatment to determine the following enzymes activities i.e. chitinase, peroxidase and polyphenol oxidase.

2.6. Measurement of enzymatic activity

Peroxidase, polyphenoloxidase and chitinase activities were determined in leaf extracts according to the method of Tuzun et al. (1989). Peroxidase activity was determined spectrophotometrically by measuring the oxidation of pyrogallol in the presence of H_2O_2 at a wave length of 425 nm. Polyphenoloxidase and chitinase activity was determined using the spectrophotometer procedure at 495 nm and 530 nm, respectively.

2.7. Mycorrhizae colonization of bean roots

Fine roots were collected from the lateral root system and fixed in formalin/acetic acid/alcohol (v/v/v) (FAA) solution. Roots were softened in 10% KOH solution for 15 min, acidified with 1M HCl for 10 min, and finally stained overnight with 0.02% Trypan blue. To assess mycorrhizal colonization, stained root segments (one cm in length) were mounted on glass slides with 50% lacto-phenol for 1–2 h prior to observation under a light stereo microscope (Leica type 020–518.500) at 200 and 400×. A minimum of 30 segments for each replicate sample was observed to assess structural colonization of AMF associated with roots. Ten segments were mounted on each slide and examined under the microscope. The distribution of mycelia, spores, vesicles and arbuscules within the root was taken as an index of colonization (Hu et al., 2015). Mycorrhizal infection was recorded within each segment to calculate the percentage of root infection as follows:

$$\text{Colonization factor (CF) percentage} = \frac{\text{number of plant fragments colonized by fungi}}{\text{total number of analyzed plant fragments}} \times 100 \quad (4)$$

2.8. The field study

The field study was conducted in the Experimental Farm of the Horticulture Department, Faculty of Agriculture Moshtohor, Benha University (Egypt) which was originally contaminated with *S. rolfsii*. This experiment was executed in a complete randomized block design for two successive seasons i.e. 2015 and 2016 with three replicates to comprise the following treatments: single inoculation with *Bacillus subtilis* (T1), combined inoculation with *Bacillus subtilis* and mycorrhiza AMF (T2), single inoculation with *Pseudomonas fluorescens* (T3), combined inoculation with *Pseudomonas fluorescens* and AMF (T4), single inoculation with AMF (T5), application of Vitavax- 200 fungicide (T6) and the control treatment (T7), The experimental plot was 10.5 m^2 (3 m × 3.5 m), each comprised 3 rows and 16 holes per row, each hole received 3 seeds. Six hundred grams of *Rhizobium leguminsarium* were mixed thoroughly with 50 kg soil and then placed in seed holes before coverage. An inoculum of AMF was incorporated within the top 20 cm of soil rows at a rate of 100 g/row.

Bean seeds were inoculated with *B. subtilis* or *P. fluorescence* by coating the seeds with a 100 mL bacterial cell suspension kg^{-1} seeds (about 2.2×10^8 cfu mL^{-1}) for 5–10 min mixed with 10% of Arabic gum solution as an adhesive agent, and then dried in the dark at room temperature. The additional suspension was added through soil drench after the emergence of two leaves to increase the rate of colonization of the bacterial cells. In case of the fungicide treatment, seeds were dressed by Vitavax-200 at a rate of 3 g kg^{-1} seed. All plots received N as ammonium sulfate fertilizer at a rate of 48 kg N ha^{-1} and 100 kg K ha^{-1} in the form of potassium sulfate after seedling emergence. From T_1 to T_5 treatment, plots were amended with a half dose of the recommended P in the form of rock phosphate i.e. 15 kg P ha^{-1}, while T_6 and T_7 treatments received the full dose in the form of calcium superphosphate (65.5 g P kg^{-1}). All the agricultural practices were followed as recommended by the Egyptian Ministry of Agriculture. Disease assessments of pre- and post-emergence damping-off as well as survived plants were recorded at 15, 30 and 60 days after planting, respectively on five plants randomly selected from each plot. At the physiological maturity growth stage, the whole plants were removed gently from soils to avoid root damage.

2.9. Plant analyses

Collected plant samples from the field study were separated into roots, shoots and green pods, oven dried at 70 C for 48 h, weighed, ground, and sieved to pass through a 2-mm micro-mill. Plant materials, 0.4 g portion each, were taken from each plant segment and digested using a mixture of concentrated sulfuric (H_2SO_4) and perchloric ($HClO_4$) acids. Total P and Fe were determined in plant digests using Atomic Absorption Spectrophotometer 210 VGP.

2.10. Statistical analysis

The obtained data were statistically analyzed using the PASW 18 statistical software through the analysis of variance (ANOVA) and least significant difference (LSD) at 0.05 probability level. Graphs were plotted using SigmaPlot10 software.

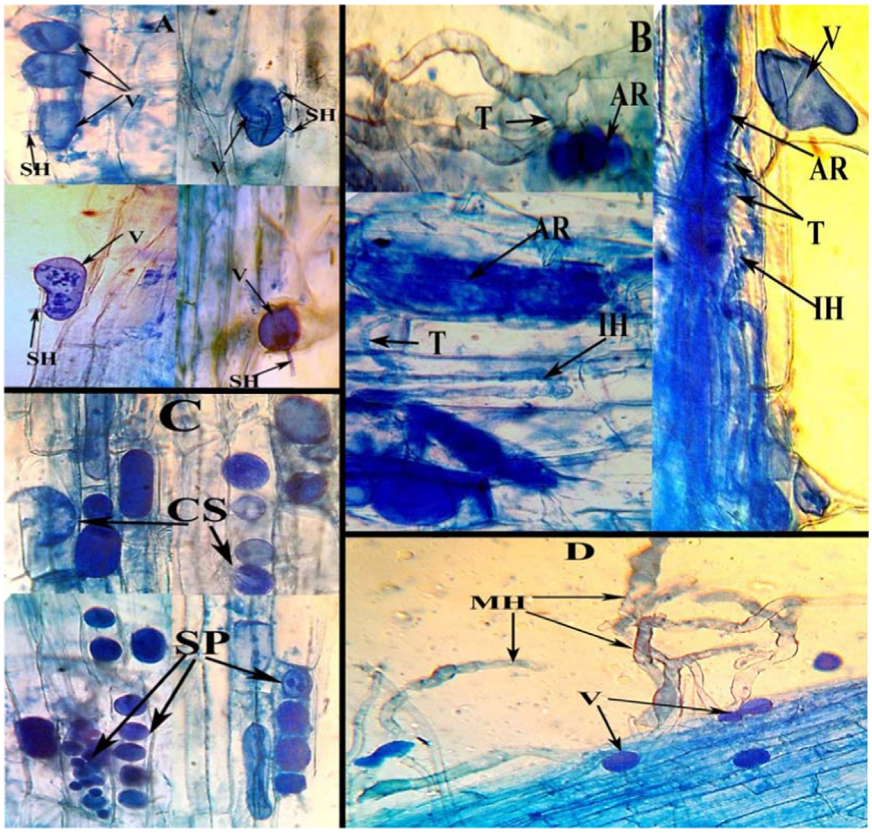

Fig. 1. Photomicrographs of structural colonization of AMF in bean roots. (A) Vesicles (V) and subtending hypha (SH) ×400. (B) intraradical hypha (IH), Arbuscule (AR), trunk (T) and vesicles (V) ×400. (C) Spore (SP), crushed spore (CS) and Intact spore (IS) ×400. (D) mycelium hyphae (MH) and vesicles (V) ×200.

3. Results

3.1. The greenhouse study

Colonization of fine bean roots by AMF is illustrated in Fig. 1(A–D) i.e. vesicles (Fig. 1A), intraradical hyphae and arbuscules (Fig. 1B), spores (Fig. 1C) and mycelium hyphae (Fig. 1D). It is worth to mention that the percentage of the calculated colonized factor in plants inoculated with *Glomus* spp in combination with either of *P. fluorescens* or *B. subtilis* did not significantly exceed than those calculated for plants inoculated solely with *Glomus* spp (Table 1). Results also reveal that both fresh and dry weights of common beans were higher in the plants inoculated with bio-agents compared with the non-inoculated ones. The dual (combined) inoculations, especially the combined treatment *B. subtilis* + AMF resulted in further significant superiority over the single ones. However; such increases did not markedly surpass the increases attained by the fungicide treatment. All the bio-treatments i.e. *B. subtilis* (T1), *B. subtilis* + AMF (T2), *P. fluorescens* (T3), *P. fluorescens* + AMF (T4) and AMF (T5) increased the activities of the investigated enzymes i.e. chitinase, peroxidase and polyphenol oxidase as compared with the control treatment or even with the fungicide one (Fig. 2). Such increases were more pronounced due to the dual bio-treatments than single ones. These results indicate the potentiality of using the investigated bioagents to control *S. rolfsii* pathogen in soils of low available P.

Table 2 shows that the percentages of pre-, post and plant survival were significantly higher for the plants inoculated with bioagents compared with the non-inoculated control ones. The mixed bio-treatments i.e. *B. subtilis* + AM (T2) and *P. fluorescens* + AMF (T4) recorded higher values in the above-mentioned parameters compared with those attained by single inoculations i.e. *B. subtilis* (T1), + *P. fluorescens* (T3) or AM (T5). On the other hand, the increases in pre-, post and plant survival seemed to be comparable between the two growing seasons.

Table 1
Bean survival (%) and its growth parameters as affected by the different bio-treatments and Vitavax-t.

Treat	Root wt (g plant^{-1})		Shoot wt (g plant^{-1})		Damping off		Survival plant %	Colonization of AMF, %
	Fresh	Dry	Fresh	Dry	Pre%	Post%		
T1	7.24 ± 0.32 b	2.65 ± 0.18 bc	32.48 ± 1.89 b	9.69 ± 0.31d	20.00 ± 11.54 b	20.00 ± 0.00 b	60.00 ± 11.55 a	00.00 ± 0.00 b
T2	8.27 ± 1.53ab	3.29 ± 0.19 a	51.02 ± 5.80 a	13.17 ± 2.16ab	13.33 ± 11.54 b	13.33 ± 11.54 b	73.33 ± 11.54 a	75.00 ± 5.00 a
T3	7.52 ± 1.65 b	2.86 ± 0.34abc	36.55 ± 2.86 b	11.65 ± 0.75bc	20.00 ± 0.00 b	13.33 ± 11.55 b	66.67 ± 11.55 a	00.00 ± 0.00 b
T4	7.83 ± 0.47ab	3.23 ± 0.26 a	46.51 ± 7.82 a	13.52 ± 0.22 a	13.33 ± 11.55 b	13.33 ± 11.55 b	73.33 ± 11.55 a	76.67 ± 2.51 a
T5	7.39 ± 1.50 b	2.90 ± 0.71ab	37.48 ± 4.99 b	10.89 ± 1.24 cd	20.00 ± 0.00 b	20.00 ± 0.00 b	60.00 ± 0.00 a	77.67 ± 2.51 a
T6	9.31 ± 0.51 a	3.05 ± 0.36ab	49.67 ± 2.85 a	14.32 ± 0.97 a	13.33 ± 11.55 b	20.00 ± 0.00 b	66.67 ± 11.55 a	00.00 ± 0.00 b
T7	5.03 ± 0.34c	2.35 ± 0.37c	22.11 ± 6.67c	7.46 ± 0.50 e	40.00 ± 0.00 a	40.00 ± 0.00 a	20.00 ± 0.00 b	00.00 ± 0.00 b

Treatments: T1: bacillus, T2: combined inoculation with mycorrhizae and bacillus, T3: Pseudomonas, T4: combined inoculation with mycorrhizae and Pseudomonas, T5: mycorrhizae, T6: fungicide (Vitavax-thiram), T7: control. Different letters on bars indicate significant difference between treatments at $P < 0.05$.

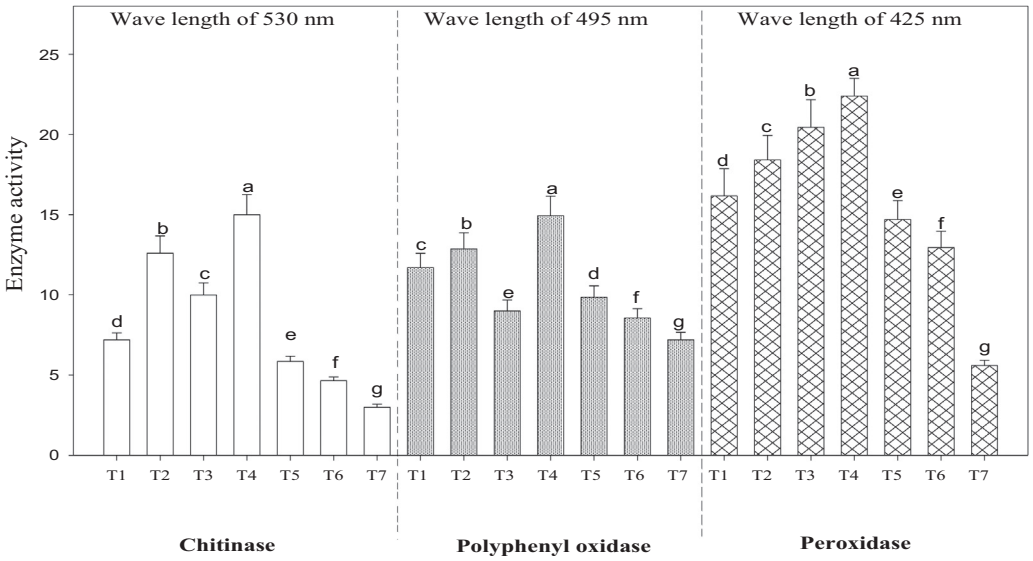

Fig. 2. Enzyme activities as affected by the different bio-treatments and Vitavax-t. Chitinase activity was expressed as mM N-acetyl glucose amine equivalent released /gram fresh weight tissue /60 min. Peroxidase activity was expressed as the change in absorbance (O.D) / minute/gram fresh weight. PPO: Polyphenoloxidase activity was assayed as the change in absorbency (O.D) /minute/gram fresh weight. Different letters on bars indicate significant difference between treatments at $P < 0.05$.

3.2. The field experiment

3.2.1. Effect of the different bio-treatments on plant survival

Analysis of variance reveals that the investigated bio-treatments increased significantly the root biomass ($F = 5.155$, $P < 0.001$) as well as the straw ($F = 13.397$, $P < 0.001$) and green pod yields of common beans ($F = 5.034$, $P = 0.001$). Moreover, these parameters i.e. root biomass ($F = 18.071$, $P = 0.001$), the yields of both straw ($F = 19.602$, $P < 0.001$) and green pod ($F = 4.88$, $P = 0.035$) were significantly higher in the second growing season than those attained in the first growing one. On the other hand, no significant interactions were detected between bio-treatments and the season of growing plants (root biomass ($F = 0.264$, $P = 0.949$), straw yield ($F = 0.368$, $P = 0.893$) and the pod yield ($F = 1.190$, $P = 0.340$)). According to Fig. 3, single bio-treatments i.e. *B. subtilis* (T1), *P. fluorescens* (T3) and AMF (T5) increased significantly root biomass, straw and pod yields compared with the control treatment (T7) (Fig. 1). The combined-inoculation treatments i.e. *B. subtilis* + AMF (T2) and *P. fluorescens* + AMF (T4) caused further significant increases in such growth parameters and yield components. It is worthy to mention that the green pod yield obtained from the combined bio-treatments exceeded those obtained from the fungicide treatment; however, such increases seemed to be insignificant (T6).

3.2.2. Effects of the different bio-treatments on P-uptake

Analysis of variance reveals that P uptake by plants increased significantly due to inoculation by the investigated bioagents ($F = 27.856$, $P < 0.001$). Such increases varied significantly between the two seasons of growing plants ($F = 19.208$, $P < 0.001$). The interaction between bio-inoculations and the season of growing plants seemed also to be significant ($F = 4.763$, $P = 0.002$). As shown in Fig. 4, all single inoculants improved significantly the uptake of P by bean plants compared with the control treatment. The uptake of P by plants inoculated with either *P. fluorescens* or *B. subtilis* did not vary significantly between the first and the second growing seasons; however, P-uptake by AMF was significantly higher in the second growing season compared with the first growing one. Generally, increases in P uptake in the second growing season followed the sequence of AMF > *P. fluorescens* > *B. subtilis*. Dual inoculations with either *B. subtilis*, AMF or *P. fluorescens* + AMF led to further significant P-uptake exceeding those recorded for the fungicide-treated plants (especially in the second growing season).

Concentrations of P within the different bean parts followed the general trend of P uptake. Generally, the highest concentrations of P were found in plant pods. Plants treated with the fungicide exhibited lower significant concentrations of P-concentrations in straw while, on the other hand, higher significant P-concentrations in roots and green pods. Meanwhile, non-inoculated control plants reserved relatively higher P-concentrations in the straw comparable with the corresponding P concentrations in roots and pods.

3.2.3. Effects of the different bio-treatments on Fe uptake

Analysis of variance reveals that Fe uptake increased significantly when plants were inoculated with the bioagents under study ($F = 20.109$, $P < 0.001$). There were also significant increases in Fe-uptake in the second growing season compared with the first one ($F = 23.600$, $P < 0.001$). The interaction between bioagents and the season of growing plants was of no significant effect on Fe uptake by common beans ($F = 0.550$, $P = 0.766$). The highest Fe uptake was recorded for

Table 2
Effect of the different bio- treatments on the bio-control indexes for survival plant (BISP).

Treatments	1st season			2nd season		
	-% Pre	% -Post	Plant survival%	-Pre %	% Post-	Plant survival%
T1	16.67 ± 2.09 b	16.67 ± 2.85b	66.67 ± 3.61 a	22.22 ± 1.21ab	15.28 ± 1.32 b	62.50 ± 0.00 a
T2	18.05 ± 2.22 b	14.58 ± 1.08b	67.36 ± 4.49 a	20.14 ± 4.34b	8.33 ± 0.61 b	71.53 ± 4.89 a
T3	16.67 ± 2.51 b	11.11 ± 1.20b	72.22 ± 6.37 a	24.30 ± 3.05ab	11.81 ± 1.20 b	63.89 ± 2.70 a
T4	13.19 ± 1.31 b	11.81 ± 2.70b	75.00 ± 4.17 a	16.67 ± 2.04b	14.58 ± 2.54 b	68.75 ± 3.05 a
T5	15.97 ± 2.41 b	16.67 ± 2.34b	67.36 ± 3.36 a	18.75 ± 3.61b	10.42 ± 1.15 b	70.84 ± 3.61 a
T6	11.11 ± 3.24 b	13.89 ± 3.18b	75.00 ± 2.08 a	13.89 ± 1.34b	15.28 ± 2.24 b	70.84 ± 4.54 a
T7	39.58 ± 2.09 a	28.47 ± 3.18a	31.93 ± 1.18 b	35.42 ± 3.62a	28.47 ± 2.41 a	36.11 ± 2.24 b

Treatments: T1: bacillus, T2: combined inoculation with mycorrhizae and bacillus, T3: Pseudomonas, T4: combined inoculation with mycorrhizae and Pseudomonas, T5: mycorrhizae, T6: fungicide (Vitavax-thiram), T7: control. Different letters on bars indicate significant difference between treatments at $P < 0.05$.

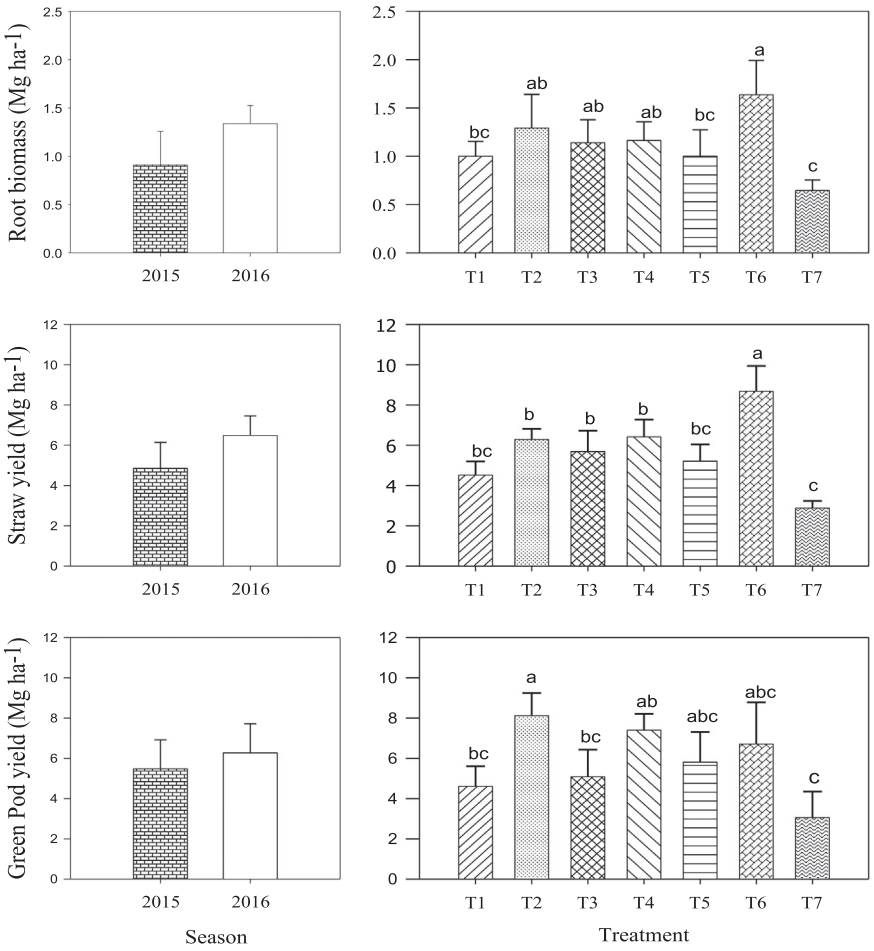

Fig. 3. Root biomass, straw and green pod yields of common beans as affected by the different bio-treatments i.e. *Bs* (T1), *Bs* + Mr (T2), + *Ps* (T3), *Ps* + Mr (T4), Mr (T5) as well as the fungicide (T6) and the control treatment (T7). Different letters on bars indicate significant difference between treatments at $P < 0.05$. Bars are standard deviations.

plants treated with either of the fungicide (T6), *B. subtilis* + AMF (T2), *P. fluorescens* (T3) and *P. fluorescens* + AMF (T4) with no significant differences among these treatments (Fig. 4). On the other hand, the lowest Fe uptake was recorded in the control plants (T7). It seems that the dual inoculations with bioagents were more efficient in increasing the uptake of Fe by the bean plants than the single ones did, in spite of that, their effect didn't exceed the effect of the fungicide treatment. Moreover, Pseudomonas inoculation, either solely or in combination with mycorrhiza seemed to be the most effective bioagent in increasing Fe uptake by plants (Fig. 5).

Concerning Fe distribution within the different plant parts, the highest Fe contents were recorded in roots followed by shoots and finally green pods of plants inoculated with *P. fluorescens* (T3), while the last ones were recorded in roots and shoots of plants inoculated with AMF (T5). Inoculation with *B. subtilis* (T1) reduced significantly Fe content in plant roots and shoots compared with the control (T7) plants; however, the corresponding values were still higher than AMF (T5). Moreover, the combined inoculation between AMF and either of *B. Subtilis* (T1) or *P. fluorescens* (T3) reduced Fe content in roots, shoots and green pods compared with the single ones (in absence of mycorrhiza) i.e. *B. subtilis* + AMF (T2) and *P. fluorescens* + AMF (T4).

It is worth to mention that Fe contents decreased significantly in plants inoculated with *B. subtilis* (T1), *P. fluorescens* + AMF (T4), AMF (T5) and the control treatment (T7) during the second growing season compared with those attained in the first growing one. On the other hand, Fe content increased significantly in plants inoculated with *B. subtilis* + AMF (T2) during the first growing season while decreased significantly in the second growing one. No significant changes were detected in Fe concentrations in plants inoculated with *P. fluorescens* (T2) between the two growing seasons.

4. Discussion

Single inoculations of common beans with either mycorrhizae or plant growth promoting Rhizobacteria (PGPR) decreased significantly the mortality of common beans while increased, at the same time, plant dry weights compared with the control ones. Combined inoculations with bioagents (*B. subtilis* + AMF or *P. fluorescens* + AMF) were more efficient in suppressing the plant pathogen than the single ones, thus increased the green pod and shoot yields beside of the root biomass. It seems that the bacterial inoculants were of no significant effect on the colonization of plant roots with AMF. These results disagree with those of Kavatagi and Lakshman (2014) and Hashem et al. (2016) who found that bacterial inoculations positively improved root colonization with AMF.

The mode of action for each of the investigated bioagents in controlling white rot fungi seemed to be different i.e. mycorrhiza increase the lignification of root cell walls as well as increase the competition with the pathogen fungi on soil nutrients and therefore reduce their growth (Sharma et al., 2004). *P. fluorescence* inhibits *S. rolfsii* probably through different mechanisms i.e. releasing antibiotics compounds (Ortíz-Castro et al., 2009; Kim, and Anderson, 2018), removing high concentrations of Fe from the medium and thus limit their availability to the pathogen fungi (Beneduzi et al., 2012) and/or inhibiting the

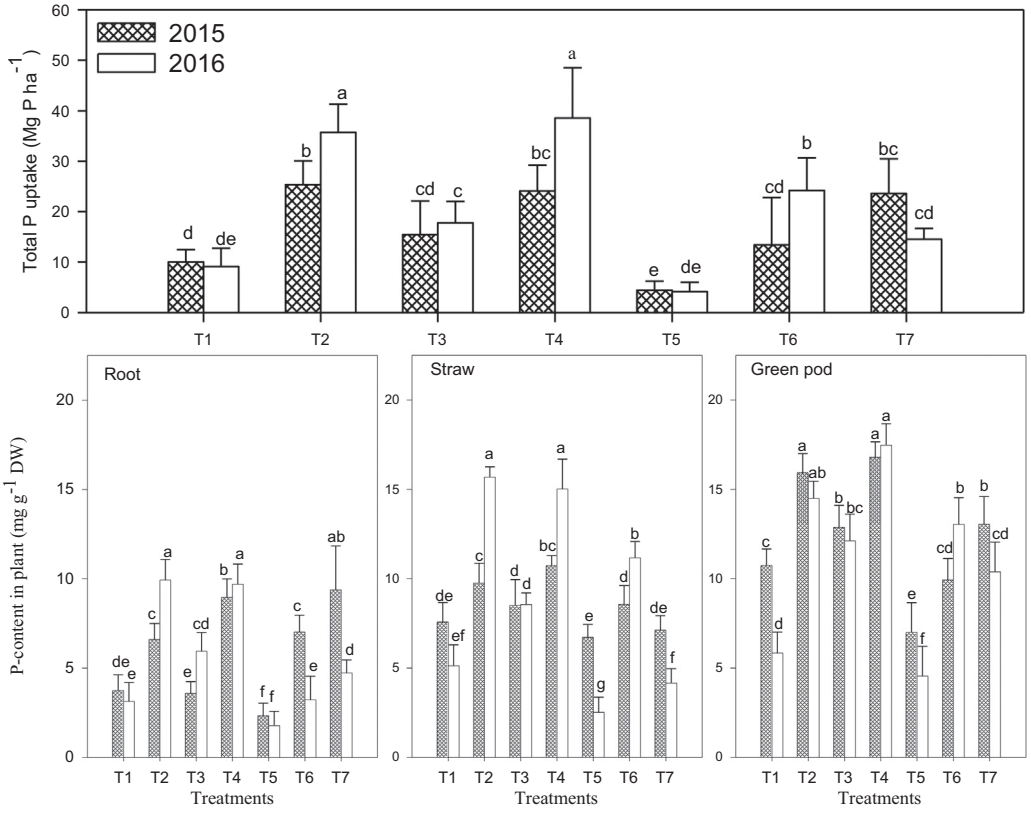

Fig. 4. Effect of the different bio-treatments on P uptake and its distribution within the different plant parts i.e. *Bs* (T1), *Bs* + Mr (T2), + *Ps* (T3), *Ps* + Mr (T4), Mr (T5) as well as the fungicide (T6) and the control treatment (T7). Different letters on bars indicate significant difference between treatments at $P < 0.05$. Bars are standard deviations.

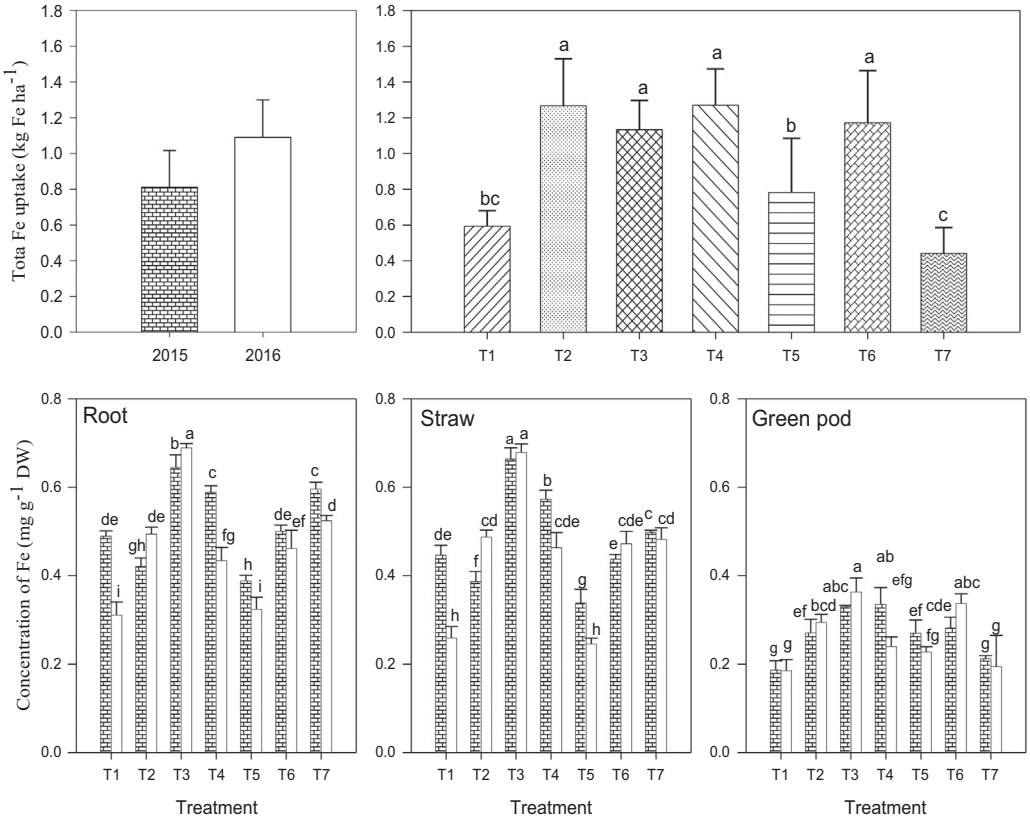

Fig. 5. Effect of the different bio-treatments on Fe uptake by common bean plants. T1: *Bs*, T2: *Bs* + Mr, T3: *Ps*, T4: *Ps* + Mr, T5: Mr, T6: fungicide and T7: control. Different letters on bars indicate significant difference between treatments at $P < 0.05$. Bars are standard deviations.

growth of mycelial of *S. rolfsii, B.* subtilis produces antifungal antibiotics (Stein, 2005), which inhibit the mycelial growth and sclerotial germination (Muhammad and Amusa, 2003). The values of straw and pod-yields obtained from the second growing season were significantly higher than the corresponding ones obtained from the first growing one. These results indicate that the used bio-treatments could successfully control the infection of common beans with *Sclerotium rolfsii*, rather than increasing the plant tolerance against the attack of the plant pathogens.

All the studied bio-treatments induced the activities of chitinase, peroxidase and polyphenyl oxidase (PPO) enzymes as compared with the control or even the fungicide treatment. Such increases were significantly higher in plants inoculated with the combined bio-treatments rather than the single ones. Chitinase enzyme probably hydrolyzed the chitin of the fungal cell wall thereby soil microbes utilize it as a source of energy (Hamid et al., 2013). Peroxidase is considered as an isoenzyme in lignification (Kawano, 2003; Shigeto et al., 2017) beside it catalyzes reactive oxygen species (ROS) (Kawano, 2003; Choudhury et al., 2017) which mediates the cellular defense in plants against microbes (Choi et al., 2007). Polyphenol oxidase PPO is another plant defense enzyme that resistant plant infection with *S. rolfsii* (Bhagat and Chakraborty, 2010). This enzyme was observed in relatively high concentrations in the PGPR-pretreated plants ± pathogen infection (Babu et al., 2015). Probably, it decreases the bioavailability of Fe (Cercamondi et al., 2013) and this might suppress the growth of the pathogen. These results agree, to some extent, with those of Al-Askar and Rashad (2010) and Abdel-Fattah et al. (2011) who found significant increases in activities of PPO in the plants infected with pathogens which were inoculated with mycorrhiza.

The enhancement in plant growth might be the net result of improving nutrient uptake and stimulating hormones (Berg, 2009; Nagargade et al., 2018; Verma et al., 2018). All seeds were inoculated with *Rhizobium* sp. and this could partially substitute the mineral nitrogen fertilizers through biological nitrogen fixation (Yildirim et al., 2011; Rosier et al., 2018). Also, a starter dose of mineral nitrogen fertilizer was applied to all seedlings in the field to induce rhizobium colonization to plant roots. On the other hand, the total amounts of P in the studied soil were relatively high while the available P content seemed to be low and insufficient to meet the plant needs. This probably took place due to the successive additions of P fertilizers to the investigated soil through several years. These fertilizers were soon fixed in soil after addition (Frossard et al., 2011; Alshaal et al., 2019). Limited supplementary inputs of P, in the form of rock phosphate, were applied to the investigated soils prior to each growing season to enrich them with P. Accordingly, the used bioagents, which were also biofertilizers that live symbiotically with the host plants, are thought to increase the availability of P in the soil and improve P uptake by the grown plants.

Single inoculations with either AMF or PGPR bacteria improved P-uptake; moreover, the combined inoculations resulted in further significant increases in P uptake by plants. Such increases might be related to the organic acids and phosphatase released by mycorrhizae or through the production of organic acids by PGPR which chelated the cations bounded to phosphate thereby increased P availability in soil (Kuhad et al., 2011; Rosier et al., 2018). Furthermore, *B. subtilis* can produce phytase (Kammoun et al., 2012) which is capable of hydrolyzing the organic phosphate found in soil and thus increasing the available concentrations of inorganic P (Singh and Satyanarayana, 2011). Increasing P availability in soil led to concurrent increases in P uptake by the grown plants, consequently, P-concentrations increased within the different plant parts. During the first growing season, relatively high concentrations of P were found in plants inoculated with *B. subtilis,* AMF, fungicide and the control ones. These concentrations might be a resultant of the physiological and biochemical changes occurred in the infected plants which increased P concentrations at the infected parts to be transported to the parasitic pathogen fungi (Walters, 2011). Another possible explanation is that plants maintained high P concentrations which can be utilized by the infected plant parts in repairing the damaged cells. During the second growing season, high assimilation rates of P might take place in plants, thus the absorbed P was more likely involved in the plant growth rather than accumulation in plant tissues as inorganic P. In this aspect, it was reported that the assimilation of nutrients dominates in the apoplast of healthy host plants rather than infected plant parts (Rico and Preston, 2008). On the other hand, values of the P uptake by *B. sitlus* + AMF, *P. fluorescens* and *P. fluorescens* + AMF were significantly higher in the second growing season than in the first one. This probably indicated that mixed inoculations as well as the single one with *P florescence* probably reduced, to a great extent, the consequences of the pathogen invasion in common beans. It is thought that high assimilation rates of P probably occur in plants inoculated with either mycorrhizae or *B. sitlus* during the second growing season; however, the increases that occurred in plant growth during this season compared with the first one might result in further dilution in concentrations of P within the different plant parts. Accordingly, P concentrations within the different plant parts were higher in the second growing season than those detected in the first one.

Inoculating common beans with either of *Bacillus subtilis*, *Pseudomonas florescence* or mycorrhiza solely, or in combinations (*B. subtilis* + AMF and *P. fluorescens* + AMF) increased significantly Fe uptake by plants compared with the non-inoculated ones (T6). Such increases were more pronounced during the second growing season than in the first growing one. This is probably because of the increases that took place in plant growth during the second growing season compared with the first one. There is no doubt that Fe is an essential nutrient for plant growth; however, *S. rolfii* can release oxalic acid to the soil rhizosphere during plant infection (Schmid et al., 2010) which might increase the solubility and availability of soil Fe for the plant pathogen (Tang et al., 2011). Thus, the infected plants might suffer from Fe deficiency. On the other hand, siderophores released by either *B. subtilis* (Zawadzka et al., 2009) or *P. fluorescence* (Reimmann, 2012) might also account for chelating soil-Fe. Such mechanism requires specific membrane-bound receptor to be taken up by soil biota (Kumar and Ashraf, 2017) which might not be found in the pathogen, thus, reduce the Fe availability for the pathogen. It was reported that *P. fluorescence* can obtain Fe from the siderophores not only produced by itself but also those produced by other microorganisms (Cornelis, 2010). Generally, inoculated plants with bioagents compete on soil Fe with the pathogen and this might effectively suppress its infection for the grown plants. Furthermore, the absorbed Fe can activate the enzymes responsible for controlling plant pathogens (Dordas, 2008) and stimulates antibiotics which are released by soil bacteria to control plant pathogens (Parewa et al., 2018). The dilution effect was more pronounced in plants inoculated with mycorrhizae; yet, Fe content in root and shoot was lower than the corresponding ones of the control or the fungicide treatment. Moreover, AMF could not utilize effectively Fe bounded to the microbial siderophores (Leyval and Reid, 1991; Winkelmann, 2017).

5. Conclusion

The synergistic effects between the inoculants PGPR and AMF might effectively control stem rot infection in common beans when soils are low in their available P. Such effects exceed the corresponding effects of single inoculations with either PGPR or AMF. This might take place through improving the uptake of P by the grown plants, hence enhancing plant growth and pod yield. Results also reveal that increasing the uptake of Fe by plants inoculated with *P.* fluorescence might be an effective process in minimizing its availability for *S. rolfii* thereby suppressing the infection of common beans with the plant pathogen; however it is difficult to say that such a mechanism is not the sole one responsible for the bio-control of the stem rot pathogens with *B. subtilis*. Thus, the synergistic effects between the inoculants PGPR and AMF can

improve the nutritional status of the infested plants and therefore stimulate the induced resistance towards soil-borne pathogens. Such an approach can minimize soil pollution through fertilizer and/or pesticide inputs.

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