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Effectiveness of Combined Inoculation of Rhizobacteria and Arbuscular Mycorrhizal Fungi on Wheat Performance

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PLANT growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi could be employed in cereal crops for growth promotion, nutrient uptake and as an alternate source of inorganic fertilizer of crops. PGPR research endeavours employed for crop enhancement have recently received great attention from researchers with thousands of studies being conducted. A growth-chamber experiment was designed to evaluate the interactive effects of PGPR and AM fungi on the growth of two different American wheat cultivars (advanced and forefront) and the bacterial populations in the wheat rhizosphere. Confocal laser scanning microscope was used to show the colonization of *Bacillus subtilis* on the roots of wheat cultivars as the response of AM fungi inoculation. Compared to the advanced cultivar, the fresh and dry weights of the shoot for the forefront wheat cultivar increased by 185% and 184%, respectively. The dual inoculation treatment by AM fungi and *B. subtilis* led to an increase in the shoot fresh weight by 69.1% and 207% and an increase in shoot dry weight by 38.9% and 169.1% in the forefront and advanced wheat cultivars, respectively. Moreover, the total number of *B. subtilis* cells found on root-hair zones significantly increased when the plants inoculated by *B. subtilis* + AM fungi compared to those plants inoculated with *B. subtilis* alone. The bacterial population in the rhizosphere inoculated with AM fungi plus *B. subtilis* was 12.1 x 10^6 and 17.1 x 10^6 cfu g^{-1} dry weight of soil for forefront and advance wheat cultivars, respectively, compared to inoculation with *B. subtilis* alone (5.8 x 10^6 and 10.6 x 10^6 cfu g^{-1} dry weight of soil). The results of this study confirm the suitability of the combined application of PGPR and AM fungi to improve wheat growth.

Keywords: AMF; CLSM; PGPR; phosphorus; Mycorrhizal dependency; wheat cultivars.

1. Introduction

In organic farming scenarios, the use of organic and biological fertilizers is a sustainable alternative to high inputs of mineral fertilizers used in the traditional production systems (Wu *et al.*, 2013). Inappropriate application of mineral fertilizers in wheat production in arid regions usually results in pollution and salinization of soils and water resources. To maintain sustainable development, the use of a nonhazardous biological method to enhance plant production is an important approach. Specifically, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi have been known for their potential use in clean agriculture (Lucy *et al.*, 2004; Tennakoon *et al.*, 2019; Abd El-Azeem *et al.*, 2014; Gopi *et al.*, 2020; Habib *et al.*, 2021). The PGPR and mycorrhizae (key component of soil microbiota) plant an important role in the maintenance of plant fitness and soil health under stressed conditions (Abd El-zeem *et al.*, 2012; Vimal *et al.*, 2017; Awad *et al.*, 2022).

Beneficial bacteria found in the rhizosphere, on root surfaces, and near roots are known as PGPR (Ahmad *et al.*, 2008). The PGPR may colonize the root surface, live and reproduce in microhabitats near the root surface and enhance plant development (Kloepper, 1996; Hassen *et al.*, 2016; Abdel-Rahman *et al.*, 2021). Protection against soil-borne plant pathogens or nutrient cycling has been attributed to PGPR (Barea *et al.*, 2002). According to previous reports, PGPR are essential for plant development under nutrient-imbalance conditions. Plant development can be aided by PGPR through several processes, including improved nutrient uptake; disease control *via* production of antibiotics and siderophores, bacterial and fungal antagonistic chemicals, phytohormone synthesis, and nitrogen fixation (Almaghrabi *et al.*, 2013; Hassen *et al.*,

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2016; Mitra *et al.*, 2021; Mondal *et al.*, 2021). Also, the production of free radical-scavenging enzymes and stress-combating enzymes that can improved drought tolerance to crop plants are key features of plant-associated microbial community (Chandra *et al.*, 2021). The mechanisms by which PGPR promote plant growth are diverse, and often the beneficial effect is due to a combination of mechanisms (Bashan *et al.*, 2004). The roots of most cultivated plant species are colonized by the AM fungi, which are obligatory symbioses. Additionally, AM fungi is an abundant component of most agroecosystems, where they give many advantages to their host plant, such as improved phosphorus nutrition (Toro *et al.*, 1998), greater drought tolerance (Hashem *et al.*, 2019), and disease resistance (Jung *et al.*, 2012) and yield (Elgharably *et al.*, 2013).

During microbial processes of root colonization, AM fungi and PGPR have been shown to interact together. The PGPR can also influence AM fungi formation and function and consequently, mycorrhizas can affect PGPR populations in the rhizosphere (Barea *et al.*, 2002). PGPR have been known to aid AM fungal development by promoting spore germination and AM fungi mycelia expansion (Xavier and Germida, 2003). The most significant physiological change that occurs during AM fungi root colonization of plants is a shift in the composition of root exudates (Azaizeh *et al.*, 1995). The bacterial community changes because of this chemical shift, resulting in the mycorrhizosphere effect. According to previous reports, PGPR is involved in the development of AM fungi symbiosis with host plant growth (Marschner and Timonen, 2005). Bacteria can be discovered clinging to the AM hyphae (Bianciotto *et al.*, 1996) as well as incorporated inside the AM fungi spore walls (Walley and Germida, 1995).

Rules with a high specificity level govern relationships between the two types of microorganisms. Due to their advantages in terms of plant development and nutrient absorption, work on the application of either AM fungi or PGPR on wheat has been demonstrated (Jaizme-Vega *et al.*, 2003; del Carmen Jaizme-Vega *et al.*, 2004). Despite these findings, dual application of both types of microbes in combination on wheat is very scarce and should be deeply investigated. According to earlier results with other crops (Rodríguez-Romero *et al.*, 2005; Ramasamy *et al.*, 2011; Xun *et al.*, 2015; Mishra *et al.*, 2016), this microbial interaction might be advantageous to wheat with the goal of establishing sustainable wheat agrosystems.

This study is part of a project by the US-Egypt cooperative research, whose main goal is to develop new biotechnologies, such as the use of bio-inoculants to improve plant growth and soil health of wheat cultivation. Therefore, the goal of this study was to demonstrate the potential of combined microbial inoculation of Arbuscular mycorrhizal fungus, *Glomus intraradices*, and plant growth promoting rhizobacterium, *Bacillus subtilis*, in improving the growth of two different American wheat cultivars.

2. Materials and Methods

2.1 Microbiological and Plant Material

Two different wheat cultivars used in the current experiment namely advanced (ad) and forefront (ff) hard red spring wheat were obtained from South Dakota agricultural experiment station (South Dakota, Brookings, USA). Before cultivation, the wheat seeds were surface sterilized for one minute in 7% bleach and washed three times with sterile water.

The arbuscular mycorrhizal fungus *Glomus intraradices* was obtained from Biology and Microbiology Department, South Dakota State University, Brookings, USA. An *in vitro* system has been developed using axenic Ri T-DNA-transformed carrot (*Daucus carota* L.) root organ cultures in Petri dishes filled with minimal medium (Bécard and Fortin, 1988). The fungus spores were collected from AM root organ culture plate by mixing the medium in 10 mM sodium citrate buffer (pH 6.0) and used for inoculation.

Pure strains of *Bacillus subtilis* were used in the experiment. This strain was isolated by the biology and microbiology department, college of agriculture and biological sciences, South Dakota State University, USA. The bacterial inoculum was prepared by culturing the strain on Luria Broth (LB) medium for 48 hr at 30 $^{\circ}$C. The inoculum concentration was approximately 2.88 x 10^8 colony-forming unit (CFU) ml^{-1}. Figure 1 (a) shows the examination *of B. subtilis* microscopically, whose cells were stained using VECTASHIELD® mounted medium with DAPI H-1200 as a fluorescent dye. Additionally, the viability of *B. subtilis* was estimated using Molecular Probes' LIVE/DEAD™ BacLight™ Bacterial Viability Kits (Figure 1 b). Even in a mixed population containing a range of bacterial species, this procedure allows the rapid, accurate, and quantitative distinction between live and dead bacteria in minutes.

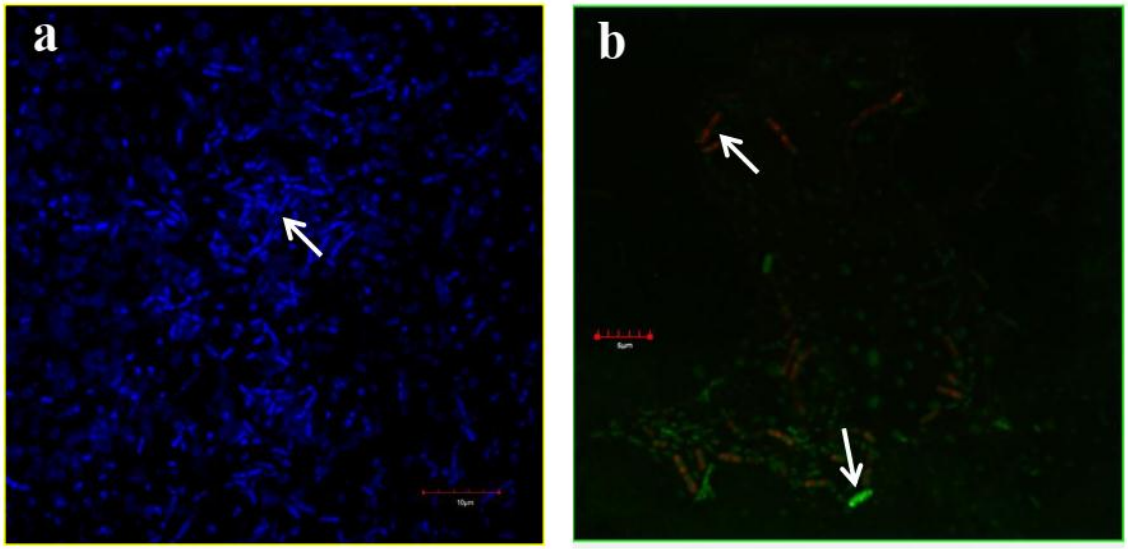

Fig. 1. Confocal laser scanning microscope images of *Bacillus subtilis* strain that used in the growth chamber experiment with different stains. Image (a) shows the shape of strain using Mounted medium. Image (b) shows the viability of *Bacillus subtilis* using LIVE/DEAD® BacLight™ bacterial viability kits. The live (green fluorescent) and dead (red fluorescent) cells may be viewed separately with fluorescein (arrowed).

2.2 BacLight™ Bacterial Viability Kits

The LIVE/DEAD BacLight™ Bacterial Viability Kits use combinations of SYTO® 9 green-fluorescent nucleic acid stain with propidium iodide, a red fluorescent nucleic acid stain. The spectrum properties of these stains vary, as does their ability to penetrate healthy bacterial cells. When applied alone, the SYTO 9 stain identifies all bacteria in a population: including those with intact and with damaged membranes. Propidium iodide, on the other hand, only penetrates bacteria with damaged membranes, resulting in a decrease in the SYTO 9 stain fluorescence when both dyes are present. Bacteria with intact cell membranes stain fluorescent green with a suitable mixture of the SYTO 9 and propidium iodide stains, whereas bacteria with damaged membranes stain fluorescent red. Consequently, a quantitative index of bacterial viability is provided by the ratio of green to red fluorescence intensities. The excitation/emission maxima for these dyes are located at 480/500 nm for SYTO 9 stain and at 490/635 nm for propidium iodide. The background remains virtually no fluorescent.

2.3 Experimental Design

The aim of the present experiment was to evaluate the effects of inoculation with plant growth promoting rhizobacterium, *Bacillus subtilis*, in combination with Arbuscular mycorrhizal (AM) fungus, *Glomus intraradices*, on the bacterial root colonization and changes of bacterial populations in the roots of wheat grown under controlled growing conditions.

A growth chamber experiment was conducted at the biology and microbiology department, college of agriculture and biological sciences, South Dakota State University, USA. Figure 2 shows the experiment setup. The glass jar was packed with a sterilized (2 h at 121°C) growth substrate of 25% organic soil, 25% perlite, and 50% sand that included 3.90 mg kg^{-1} NO_3, 40.3 mg kg^{-1} NH_4^+, and 1.0 mg kg^{-1} P (Olsen method, (Kuo, 1996). In a growth chamber, the plants were grown under the following conditions: 15.5 h photoperiod, 25 °C: 20 °C, day: night cycle, 1/3 rd of fluorescent lights active, no incandescent light and 35% humidity. The plants were irrigated with distilled water every four days when needed.

The experiment included four treatments (replicated six times): forefront (ff) or advanced (ad) wheat varieties inoculated with *B. subtilis* alone ('ff + PGPR' and 'Ad + PGPR'), and inoculation with both *Glomus intraradices* and *B. subtilis* ('ff + AM fungus + PGPR' and 'Ad + AM fungi + PGPR'). The sterilized jar was organized according to a randomized block design.

Inoculation with AM fungi was conducted at a rate of 442 spores ml^{-1} at the time of sowing. Four wheat seeds for each cultivar were immediately sown in each jar and irrigated to approximately field capacity. The seedlings were thinned to two uniform plants per jar after a couple of weeks from sowing date. The plants were harvested after 8 weeks from sowing date and growth parameters (shoot and root dry weights) were recorded.

Fig. 2. Experiment setup inside the growth chamber.

2.4 Root Sampling and Preparation

To determine the bacterial root colonization, the root sample was taken after 6 weeks from cultivation. Briefly, the roots were gently shaken to remove adhering soil particles, gently washed in deionized water in Petri dishes, separated from the shoot, and then dried on sterilized paper tissue (Neumann *et al.*, 2009). Samples were used for immediate microscopy.

2.5 Visualization of Root Colonization

To visualize wheat root colonization by the bacterium *B. subtilis* microscopically, confocal laser scanning microscopy (CLSM, OLYMPUS FV1200-FV10-ASW, Olympus Cooperation, Tokyo, Japan) equipped with 3 fluorescence channels, 3 lasers, and AOTF was used in this study. This approach was used to address numerous applications in a broad range of innovative research fields. CSLM considers the "live cell observations", with which long hours of stable measurement of weak fluorescence is required. The root samples were directly transferred to a glass slide and stained for 15 minutes using Molecular Probes Live/Dead® Bacterial Viability Kit (Backlight^{TM}). A mixture of dyes: SYTO 9 (living bacteria/green) and propidium iodide (dead bacteria/red) were used to stain the bacterial strain. Fluorescence from SYTO 9 was excited at 480-500 nm, with the emitted fluorescence measured at 500 nm, while fluorescence from propidium iodide was excited at 490-635 nm, with the emitted fluorescence measured at 618 nm. Stained cells images processed with a confocal microscope. The bacterial cells were identified as green-fluorescent cells. Images were taken and processed using CLSM image viewer program.

2.6 Soil Bacterial Population

To evaluate the effects of inoculation with AM fungus alone or in combination with PGPR on soil bacterial activity, the number of *B. subtilis* was enumerated in the rhizosphere and bulk soil (nonrhizosphere) by the dilution plate method. One gram of moist soil was diluted 10 times with 9 mL sterilized water before being serially diluted. Then, a 50-µL aliquot of the serially diluted sample was placed immediately onto the surface of the tryptic soy agar (TSA) plate. The inoculated plates were incubated at 30 °C for 3 days. The bacterial populations were expressed as colony forming units (CFU) x 10^n g^{-1} oven dried soil, where 10^n was the dilution factor (Pepper & Gerba, 2009).

2.7 Plant Phosphorus Content

Shoot and root materials were ground in liquid nitrogen, weighed, dried at 90 °C, and digested by the addition of 1 M HCl for 2 h at 95 °C. The concentration of phosphorus was analyzed using spectrophotometer at 436 nm after the addition of ammonium molybdate-vanadate solution (Ricca Chemical).

2.8 Mycorrhizal dependency

Mycorrhizal dependency or responsiveness of the two examined wheat cultivars was evaluated in terms of the ratio of the shoot dry mass of mycorrhizal (M) to the non-mycorrhizal (NM) plants as follows (Bottomley et al., 2020):

$$\frac{M - NM}{NM} \times 100$$

2.9 Statistical Analysis

Experimental data were statistically analyzed by one-way analysis of variance (ANOVA) using the SPSS 26.0 statistical software (SPSS Inc., IL, USA), and the means were compared using the Least Significant Difference (LSD) test. Values of $P \leq 0.05$ were considered to indicate significance ($n = 6$).

3. Results

3.1 Growth promotion of wheat plants

Table 1 shows the responses of the two wheat cultivars under the effect of inoculation with either AM fungi or PGPR and the combination of them.

The results revealed that the plant growth of forefront wheat cultivar is more responsive to bacterial inoculation alone than the advanced wheat cultivar. The weights of fresh and dry shoot of forefront cultivar increased by 235% and 184%, respectively, when compared to the advanced wheat cultivar. In the contrary, the response of root growth to bacterial inoculation alone was similar in both cultivars. As shown in Table 1, the plant growth (shoot fresh and dry weights and root dry weight) was positively affected by AM fungus inoculation. In dual inoculation treatment with both AM fungi and *B. subtilis*, the shoot fresh weight increased by 69.07% and 207% and the shoot dry weight increased by 38.86% and 169.12% in the forefront and advanced wheat cultivars, respectively, compared to the bacterial inoculation alone. However, the response of root growth (root dry weight) in wheat plants was similar in both cultivars.

3.2 Mycorrhizal Dependency

Table 2 shows that highly differences were noted in the degree of response of wheat cultivars to the mycorrhizal inoculation. The investigated cultivars' mycorrhizal dependence (MD) varied greatly. The values of MD were 68.6% and 259.8% for forefront and advanced cultivars, respectively. Table 2 demonstrated also that wheat cultivars varied in their shoot and root dry weight. Root weight was inversely correlated to mycorrhizal dependency. For instance, advanced wheat cultivar had smaller root dry weight and benefited more from mycorrhizal inoculation; meanwhile, the forefront cultivar having higher root dry weight had less mycorrhizal dependency (Table 2). The inoculation with mycorrhiza stimulates root and shoot growth and the root to shoot (R/S) ratio was also enhanced.

Table 1. Effect of inoculation with *Bacillus subtilis* alone or in combination with *Glomus intraradices* on the wheat shoot and root biomass (g jar^{-1}). Values are the means ± SD ($n = 6$)*.

Treatment	Shoot biomass (g)		Root biomass (g)
	Fresh weight	Dry weight	Dry weight
ff + *B. subtilis*	0.97±0.058^{b}	0.193±0.001^{b}	0.052±0.017^{a}
ff + *B. subtilis* + *G. intraradices*	1.64±0.281^{c}	0.268±0.034^{c}	0.141±0.017^{b}
ad + *B. subtilis*	0.34±0.037^{a}	0.068±0.022^{a}	0.053±0.019^{a}
ad + *B. subtilis* + *G. intraradices*	0.89±0.048^{b}	0.183±0.020^{b}	0.142±0.009^{b}

ff; forefront wheat cultivar, Ad; Advanced wheat cultivar
*Values followed by different superscripted letters in the same column are significantly different (P≤0.05)

Table 2. Mycorrhizal Dependency and dry weight of root and shoot ((g jar^{-1})) of inoculated and uninoculated wheat cultivars. Values are the means ± SD ($n = 6$)*.

Wheat Cultivar	Mean plant dry weight (g)						Mycorrhizal dependency (%)
	Without mycorrhiza			With mycorrhiza			
	Root	Shoot	R/S ratio	Root	Shoot	R/S ratio	
Forefront	0.052±0.017^{a}	0.193±0.001^{b}	0.270	0.141±0.017^{b}	0.268±0.034^{c}	0.530	68.6±0.223
Advanced	0.053±0.019^{a}	0.068±0.022^{a}	0.894	0.142±0.009^{b}	0.183±0.020^{b}	0.787	259.8±1.90

3.3 Concentration of Phosphorus in Roots and Shoots

Fig. 3 shows the concentration of P in shoots and roots of the two examined wheat cultivars as affected by the microbial treatments. The results indicated that the combined treatment with tested bacterium and AM fungus augmented the P-content in the shoots and roots of the wheat plants compared with the bacterial inoculation treatment alone. The largest increased (about 2.21-fold and 7.91-fold) of P-content in shoots and roots was observed when the dual inoculation was applied in the forefront and advanced wheat cultivars, respectively.

Soil inoculation with PGPR and AM fungi is a promising tool for integrated management systems, and numerous efforts have been employed to improve the efficiency of plants to use nutrients, particularly P (from either soil or fertilizers) through biological technology and the sustainability of the cropping systems.

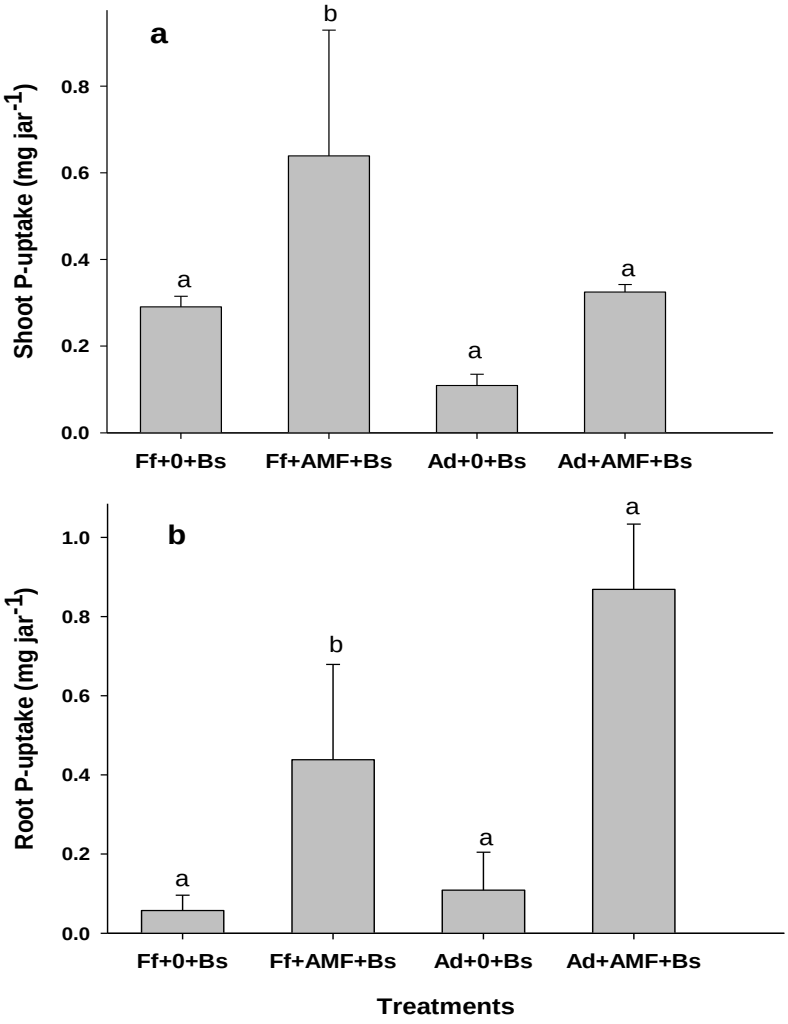

Fig. 3 Phosphorus content in the shoot (a) and root (b) of two wheat cultivars. Treatments are designed as ff + Bs, forefront wheat cultivar + *Bacillus subtilis*; ff + AM + Bs, forefront wheat cultivar + *Bacillus subtilis* + *Glomus intraradices*; ad + Bs, advanced wheat cultivar + *Bacillus subtilis*; ad + AM + Bs, advanced wheat cultivar + *Bacillus subtilis* + *Glomus intraradices.* Vertical bars represent standard deviation.

3.4 Detection of Bacillus subtilis Colonizing on Wheat Roots Using CLSM

Confocal analysis was used to demonstrate surface colonization of *B. subtilis* on 3-4 cm long wheat root longitudinal sections. The CLSM method enabled greater insight into the spatial distribution of bacterium *B. subtilis* in the rhizosphere and rhizoplane of wheat seedlings. The quality of image was considerably enhanced by using single-spot excitation to reduce background fluorescence. In addition, fully focused optical options were created, which may be merged to create a colourful extended-focus image. Figure 4 shows the *Bacillus subtilis* which was continuously connected to the surface of the root hairs (arrowed in images 4A and 4B stained by VECTASHIELD® mounted medium). The *B. subtilis* also attached the surface of AM fungi hyphae (Fig. 4E) and root surface (Fig. 4F) (stained by LIVE/DEAD® BacLight™ bacterial viability kits). The *B. subtilis* cells were found in huge counts of root hairs zones of the seedlings of two the examined cultivars when the plants dually inoculated with *B. subtilis* plus AM fungus (Fig. 4D) compared to the plants inoculated with *B. subtilis* alone (Fig. 4C).

3.5 Soil Bacterial Population

In contrast with the bulk soil (non-rhizosphere), where microbes are usually present in low counts and activity, the rhizosphere is a location of high microbial activity and composed of a wide range of exudates made available by young roots. Figure 5 shows the quantitative analysis of the bacterial population in the bulk and rhizospheric soil. Generally, the results indicated that the populations of *B. subtilis* in the rhizosphere were significantly greater than those in non-rhizosphere for the two

wheat cultivars. This finding indicated that the rhizosphere effect (root exudates) plays a crucial role in bacterial populations in this zone. The inoculation with AM fungus in combination with *B. subtilis* significantly increased the bacterial count in the rhizosphere or bulk soil compared to inoculation with *B. subtilis* alone. For examples, bacterial population revealed a considerable increase in rhizosphere inoculated with AM fungi plus *B. subtilis* with 12.13 x 10^6 and 17.09 x 10^6 cfu g^{-1} oven dry soil in the forefront and advance wheat cultivars, respectively, compared to those inoculated with *B. subtilis* alone (5.77 x 10^6 and 10.62 x 10^6 cfu g^{-1} oven dry soil).

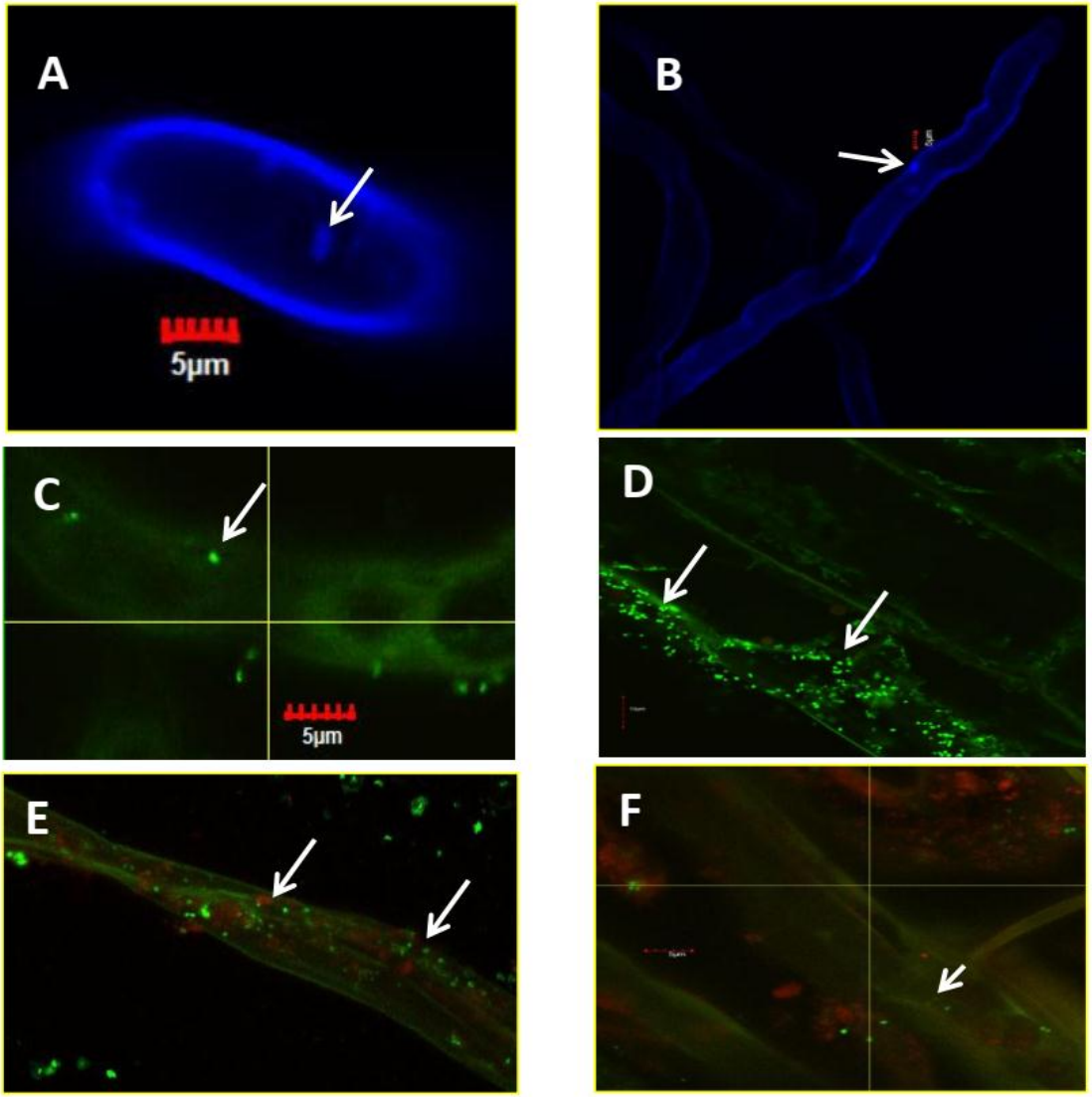

Fig. 4. Confocal laser scanning microscope images of *Bacillus subtilis* and *Glomus intraradices* strains that used in the growth chamber experiment. *B. subtilis* was continuously connected to the surface of the root hair (arrowed) in images 4A and 4B (VECTASHIELD® mounted medium). It also attached to the surface of AMF hyphae (4E) and root surface (4F) (LIVE/DEAD® BacLight™ bacterial viability kits). Images 4C shows less *B. subtilis* cells that attached the wheat root surface (wheat plant inoculated with bacteria alone) compared to image 4D shows more *B. subtilis* cells that attached the wheat root surface (wheat plant inoculated with *B. subtilis* and *Glomus intraradices* strains).

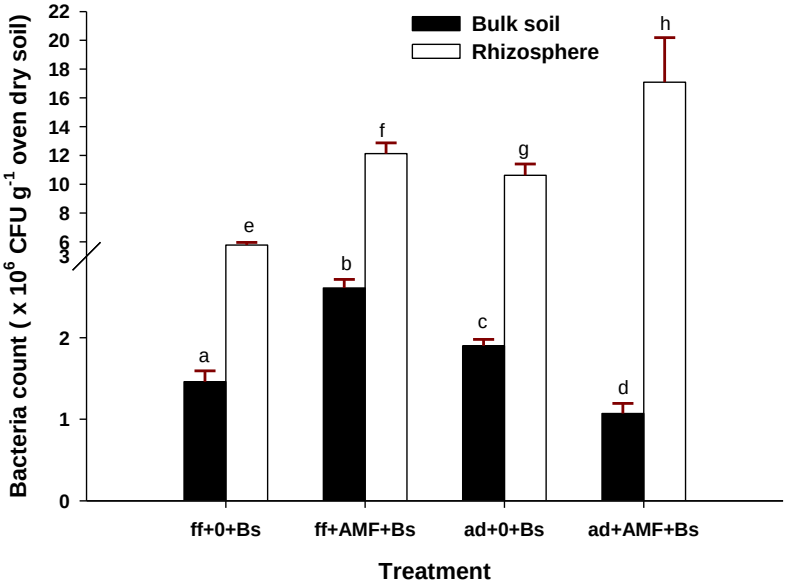

Fig. 5. Effects of *Bacillus subtilis* alone or in combination with *Glomus intraradices* on the population of bacteria in two wheat cultivars bulk and rhizosphere soil under growth chamber conditions. (Wheat cultivars, Forefront (ff) and Advance (ad)).

4. Discussion

The effects of the combined inoculation of the Arbuscular mycorrhizal fungus, *Glomus intraradices* and *Bacillus subtilis*, on two American wheat cultivars under growth chamber conditions, was investigated. In this study, the mixtures of tested mycorrhiza and PGPR strain were related to the amount of AM fungal colonization of wheat roots. On the two different wheat cultivars, clear favourite was noticed for the microbial associations and beneficial effects of the bacterial strain. The following themes were identified related to the wheat performance and improvement:

For the wheat growth promotion in the growth chamber, the results indicate that the plant growth of forefront wheat cultivar is more response to bacterial inoculation alone than that of the advanced wheat cultivar. This may be explained by the close natural relationship between *Bacillus subtilis* and forefront wheat roots. The bacterial strain that is linked with the *Glomus* species has an influence on shoot and root development. Previously, several particular effects in the *Glomus*-bacterium interaction had been described (Medina *et al.*, 2003). This finding suggested that the *Glomus* species may have varying capacities to develop external mycelium depending on the length of infected root, which can be changed because of the bacterial inoculation (Medina *et al.*, 2003). On the other hand, the production of metabolites, that involved in increasing root cell permeability and hormone synthesis by the PGPR, is the main mechanism for increasing AM colonization (Dwivedi *et al.*, 2009). In this regard, (Zaidi *et al.*, 2009) reported that *Bacillus subtilis* has demonstrated a direct contribution in the process of P solubilization alone and through the development of a synergistic relationship with AM fungi. Mycorrhizal mycelium might absorb the microbiologically solubilized P, resulting in a beneficial microbial interaction.

In the experiment with two tested cultivars, a wide range of mycorrhizal dependencies have been observed. Wheat mycorrhizal dependency increased with advanced cultivar compared to forefront. In this regard, (Azcon & Ocampo, 1981) found that when the wheat root weight increased the wheat mycorrhizal dependency decreased. It is well known that mycorrhizal dependency appears to be significantly influenced by root formation.

For phosphorus content in the roots and shoots, the present work found that inoculation with AM fungus in combination with PGPR strain increased shoots and roots P-content in two wheat cultivars compared to PGPR inoculation alone. This finding mainly attributed to increase soil phosphorus pool available by PGPR inoculation for the extraradical mycelium AM fungal hyphae (ERM) to pass on the plant. The ERM mycelium of the fungus acts as an expansion of the root structure. It absorbs nitrogen, phosphate, sulfur, and trace elements from the surrounding soil, and delivers these nutrients *via* the intraradical mycelium (IRM) to the plant (Artursson *et al.*, 2006; Mensah *et al.*, 2015). This beneficial impact on P uptake has been linked to: (i) the extraradical mycelium (ERM) exploring a greater

soil volume; (ii) enhancing P absorbing surface area through small hyphal diameter; (iii) formation of long-chained or short chain polyphosphates by mycorrhizal fungi, which lower internal inorganic P concentrations (Bücking and Shachar- Hill, 2005). Polyphosphates is involved in the storage of P in the fungal hyphae and transfer of P from the ERM to the IRM (Mensah *et al.*, 2015). In comparison to their control, which were not dually inoculated, the treatments inoculated with both AM fungus and tested bacterium significantly enhanced plant biomass and the concentration of P in the plant tissues. In this regard, using ^{32}P isotopic dilution method, (Toro *et al.*, 1997) found that the inoculation with rhizobacteria led to a release of P ions (^{31}P) from rock phosphate added or from the inaccessible indigenous P sources that were effectively absorb by the external AM mycelium. Without addition of rock phosphate, AM plants had lower specific activity ($^{32}P/^{31}P$) than did their corresponding non-mycorrhizal controls, indicating that plant was using otherwise unavailable P resources (Barea *et al.*, 2002). Additionally, (Shams El-Deen *et al.*, 2020) found that the combined inoculation with bacterial strains and AM fungi significantly increase P content in wheat plant tissues compared with the individually inoculated soil.

For detection of *Bacillus subtilis* colonizing on wheat roots using CLSM, this finding indicated that the root hairs of wheat seedlings were heavily colonized with this bacteria strain. This could be due to the mycorrhizal plant, which can alter the chemical composition of root exudates, are typically a source of nutrients to associated bacteria in the mycorrhizosphere. These findings supported with previous research indicating that a great degree of specificity occurs between bacteria associated with AM fungi (Artursson *et al.*, 2005, 2006). The bacteria, which are stimulated by species-specific fungal exudates, might be one reason for the specificity of distinct bacterial species by specific AM fungi.

Finally, colonization of plant roots by AM fungi can change bacterial populations surrounding the roots in both directly and indirectly mechanisms, affecting soil bacterial populations. Direct mechanism includes the providing of energy-rich carbon compounds resulting from assimilation of host that are transported to the mycorrhizosphere through fungal hyphae, competition for nutrients, fungus-induced pH changes in the mycorrhizosphere, and fungal exudation of other inhibitory or stimulatory compounds. Mycorrhiza-mediated impacts on host plant growth, root exudation, and soil structure are examples of indirect interactions (Johansson *et al.*, 2004). In this respect, (Bianciotto *et al.*, 1996) showed that rhizobacteria form biofilms on the AM fungi spores and hyphae, suggesting that these fungal structures are very suitable habitats for soil bacteria. The microbial community structure was observed to have modified because of mycorrhizal colonization and microbial inoculation. Understanding of these impacts as component of ecosystem processes is critical for maximizing plant growth and health in the context of long-term soil-plant system sustainability. The results confirmed the fact that the inoculation with AM fungi stimulated and change bacterial growth through change in root exudation patterns (Shahzad *et al.*, 2015).

5. Conclusions

Our study assessed the impact of plant growth-promoting rhizobacterium, *B. subtilis*, on the growth and interactions of spring wheat with arbuscular mycorrhizal (AM) fungi in growth chamber conditions. It was identified from the results of this study that forefront fresh and dry weights of shoot increased by 235% and 184%, respectively, compared to advanced wheat cultivar. The plant growth was positively affected by AM fungi inoculation. The shoot fresh weight increased by 69.07% and 207% in dual inoculation treatment in the forefront and advanced wheat cultivars, respectively, compared to bacterial single inoculation. The shoot dry weight increased by 38.86% and 169.12% in plants that treated with AM fungi and *B. subtilis* for forefront and advanced wheat cultivars, respectively, compared to bacterial single inoculation. The dual inoculation with *B. subtilis* and AM fungus increased P-content in shoots and roots compared with bacteria only. The largest increased (about 2.21-fold and 7.91-fold) P-content in shoots and roots was observed with dual inoculation treatment in the forefront and advanced wheat cultivars, respectively. On the surface of seedlings of two cultivars, *B. subtilis* cells were observed in huge numbers of root hairs regions when the plants inoculated with *B. subtilis* plus AM fungus compared to the plants inoculated with *B. subtilis* alone. The bacterial population showed a cleared increase in rhizosphere inoculated with AM fungi plus *B. subtilis*, indicating 12.13×10^6 and 17.09×10^6 cfu g^{-1} oven dry soil for forefront and advance wheat cultivars, respectively, compared to inoculated with *B. subtilis* alone (5.77×10^6 and 10.62×10^6 cfu g^{-1} oven dry soil). These results evidenced that the inoculation with AM fungus stimulated and change bacterial growth through changing root exudation patterns.

6. Declaration of competing interest

The author declares that he has no conflict of interest.

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