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Proceeding Paper

Effect of *Azospirillum brasilense* and *Bacillus subtilis* Inoculation on Durum Wheat Growth Response under Four Inoculation Methods †

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Abstract: The growth response of durum wheat (*Triticum durum*) to inoculation by two rhisospheric rhizobacteria: *Azospirillum brasilense* and *Bacillus subtilis* was evaluated using four ways of inoculation to determine the best method that gives better results. The two rhizobacteria were inoculated on LB liquid medium. Durum wheat was inoculated twice. For the first inoculation, part of the germinated seeds was directly sown on the ground and inoculated by bacterial pellet (PP) or by medium containing bacterial culture (MM). As for the other part of the germinated seeds, they were first immerged for 45 min in a medium containing the bacterial culture, after that, they were sown on the ground. For the second inoculation, it was carried out 10 days after sowing, by the same method, except for the seeds being immerged in the culture medium, which were inoculated this time by bacterial pellet (IP) or by medium containing bacterial culture (IM). After 3 weeks of growth, different plant parameters such as the fresh and dry weight of leaves and roots, the number of leaves, the length of leaves and roots and the chlorophyll levels were compared between inoculated and non-inoculated plants and according to the different inoculation methods. The results demonstrated that the inoculation of durum wheat with these two strains stimulated the growth of the plant, some parameters gave similar effects between the two bacteria and other parameters gave different effects. Similarly, the type of inoculation influenced the response of the plant to the bacterium; some types gave better results compared to others.

Keywords: durum wheat; *Azospirillum brasilense*; *Bacillus subtilis*; inoculation

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1. Introduction

In agriculture, the soil and environment are negatively affected by the incidental use of chemical fertilizers, and the researcher is confronted with the challenge of finding a more sustainable solution to help prevent climate change and preserve the fertility of the soil [1,2]. Plant growth-promoting rhizobacteria (PGPR) can be used as an alternative to chemical fertilizers [3,4]. This group of bacteria colonize the rhizosphere and enhances plant growth through direct and indirect mechanisms [5]. Wheat is a crucial crop in agriculture, it is one of the most consumed foods by the world's population, and PGPR can be used to enhance its growth and yield [6–8]; the PGPR are also able to facilitate the plant's adaptation to both biotic and abiotic stresses [9–11]. The beneficial effects of PGPRs can be significantly enhanced and improved using different inoculation methods.

Using two different PGPR strains, our work aims to test the effect of four methods of plant inoculation on durum wheat growth, to determine the most effective inoculation method.

2. Materials and Methods

Two PGPR bacterial strains *Azospirillum brasilense* and *Bacillus subtilis* were used to test the effect of their inoculation on the growth of durum wheat. The two strains were inoculated on Luria-Bertani (LB) broth medium [12].

The Cirta variety seeds of durum wheat have been sterilized and pre-germinated for 2 days. The germinated seeds are then sown in sterile ground. All the plants are inoculated twice, the first time on the day of sowing and the second inoculation after the tenth day, following the procedure described in Table 1. Overall, four different inoculation methods are used in comparison with the control.

Table 1. Different inoculation methods applied to durum wheat.

	First Inoculation	**Second Inoculation**
Control	Not inoculated	Not inoculated
(PP)	Inoculated with the pellet of the centrifuged bacterial culture.	Inoculated with the pellet of the centrifuged bacterial culture.
(MM)	Inoculated with culture medium containing bacterial culture.	Inoculated with culture medium containing bacterial culture.
(IP)	The germinated seeds were immerged and shacked for 45 min in LB medium containing the bacterial culture.	Inoculated with the pellet of the centrifuged bacterial culture.
(IM)	The germinated seeds were immerged and shacked for 45 min in LB medium containing the bacterial culture.	Inoculated with culture medium containing bacterial culture.

The same bacterial concentration is applied to different inoculations. After 21 days of growth in the greenhouse, the plants are harvested, the leaves and roots are separated and their fresh and dry matter weighed, their length is measured and their chlorophyll content is determined [13,14].

3. Results

3.1. Effect of Different Methods of Inoculation on Morphological Response of Durum Wheat

After 21 days of growth in the greenhouse, the plants were harvested, and the leaves and roots length were measured. We observed that inoculation by pellet (PP) of *Azospirillum brasilense* significantly increased the length of the leaves compared to the control (Table 2). On the other hand, inoculation by immersion of the seeds and medium containing bacterial culture (IM) of *Bacillus subtilis* significantly increased the length of leaves. However, the other inoculation methods slightly increased the length of the leaves compared to the control, except for the plants inoculated with (MM) of *B. subtilis*, which had the lowest length growth (Table 2). Regarding the effect of inoculation on the fresh weight of the leaves, inoculation with (PP) of *A. brasilense* had a significantly high fresh weight, but in plants inoculated with *B. subtilis*, it was inoculation with (PP) or (IM) that gave a significantly higher weight compared to the control. On the other hand, the dry weight of the leaves was not affected by the inoculation or by the different inoculation methods, except for the plants inoculated with (PP) of *B. Subtilis*, where their dry weight presented a highly significant effect (Table 2). Similarly, the roots fresh weight and dry weight were not affected by the different inoculation methods in the presence of each of the two strains (Table 2).

Table 2. Morphological parameters of durum wheat were affected by different methods of inoculation. Values represent the means ± standard deviation (n = 15). Different letters represent significant differences between treatments using two-way ANOVA with Fisher's LSD multiple comparison post-test at p = 0.05.

Bacterial Strains	Inoculation Method	Leaves Length (cm)	Roots Length (cm)	Leaves Fresh Weight (g)	Leaves Dry Weight (g)	Roots Fresh Weight (mg)	Roots Dry Weight (mg)
Not inoculated	Control	33.93 ± 2.70 bc	19.29 ± 2.49 abc	1.85 ± 0.45 def	0.22 ± 0.05 b	710.98 ± 286.26 a	65.00 ± 14.86 ab
Azospirillum brasilense	(PP)	37.25 ± 1.86 a	19.83 ± 1.80 ab	2.38 ± 0.27 a	0.25 ± 0.04 b	571.00 ± 63.89 ab	63.22 ± 12.93 ab
	(MM)	34.77 ± 2.39 b	15.15 ± 2.12 e	1.94 ± 0.35 bcd	0.21 ± 0.05 b	439.41 ± 162.03 b	50.11 ± 10.15 c
	(IP)	34.31 ± 1.84 bc	16.54 ± 2.96 de	1.59 ± 0.48 f	0.19 ± 0.06 b	450.65 ± 102.37 b	46.16 ± 12.03 c
	(IM)	35.14 ± 2.51 b	17.86 ± 2.07 bcd	1.83 ± 0.48 def	0.20 ± 0.06 b	442.00 ± 139.39 b	50.44 ± 11.24 c
Bacillus subtilis	(PP)	35.20 ± 2.01 b	19.07 ± 3.45 abc	2.15 ± 0.25 abc	0.44 ± 0.24 a	707.64 ± 231.88 a	68.57 ± 25.67 ab
	(MM)	32.93 ± 2.25 c	16.13 ± 2.83 de	1.63 ± 0.29 ef	0.20 ± 0.03 b	467.39 ± 82.23 b	57.45 ± 20.95 bc
	(IP)	35.00 ± 2.14 b	20.07 ± 3.01 a	1.90 ± 0.43 cde	0.24 ± 0.06 b	623.60 ± 181.71 a	70.50 ± 17.53 a
	(IM)	37.44 ± 1.94 a	17.44 ± 1.42 de	2.22 ± 0.29 ab	0.24 ± 0.03 b	672.52 ± 102.23 a	56.93 ± 8.64 bc

It was observed that *A. brasilense* and *B. subtilis* accelerated leaf emergence by increasing leaf number (Figure 1). Both strains generated plants with a number of leaves between ten to twelve leaves. This class of plants did not appear in non-inoculated plants (Control). In plants inoculated with *A. brasilense* under the (IP) method, we observed that 38.46% of plants had ten to twelve leaves. In addition, when the same strain was inoculated by pellet (PP), 100% of the plants had between seven and nine leaves, and there was an absence of plants having four to six leaves (Figure 1). However, inoculation with *B. subtilis* showed that all inoculation methods generated plants with ten to twelve leaves, whereas this class was not present in the control. Comparing the two bacteria, we noted that inoculation with the (IM) method for both strains gave two types of plant class: (i) plants that reached the stage of seven to nine leaves and (ii) plants that reached the stage of ten to twelve leaves, with the absence of the four to six leaves class (Figure 1).

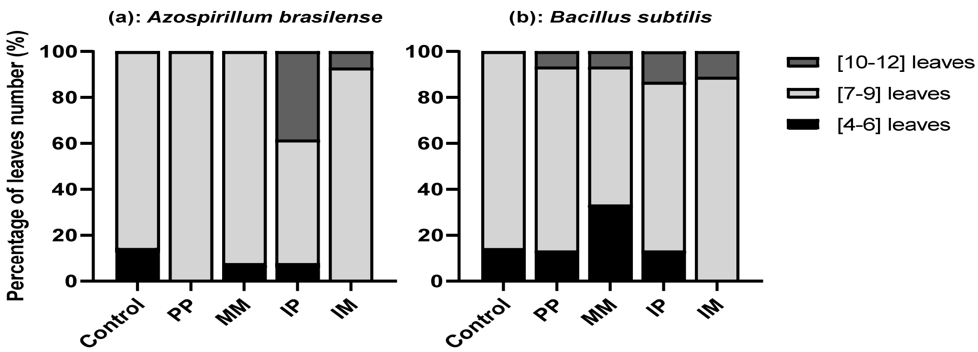

Figure 1. Number of leaves affected by different inoculation methods. Plants were inoculated by (**a**) *Azospirillum brasilense* or (**b**) *Bacillus subtilis*. For each inoculation, different bars presented the percentage of plants that developed a specific number of leaves compared to the total number.

3.2. Chlorophyll Level Effected by Different Methods of Inoculation

The plants inoculated with the (IM) method in the presence of *A. basilense* or *B. subtilis* showed a significant increase in the levels of chlorophyll a and b as compared to the control (Figure 2). PGPR has been shown to also have a positive effect on plant chlorophyll levels, especially under stress conditions [15]. However, chlorophyll a showed the highest increase compared to chlorophyll b. For total chlorophyll, the same result was observed (Figure 2).

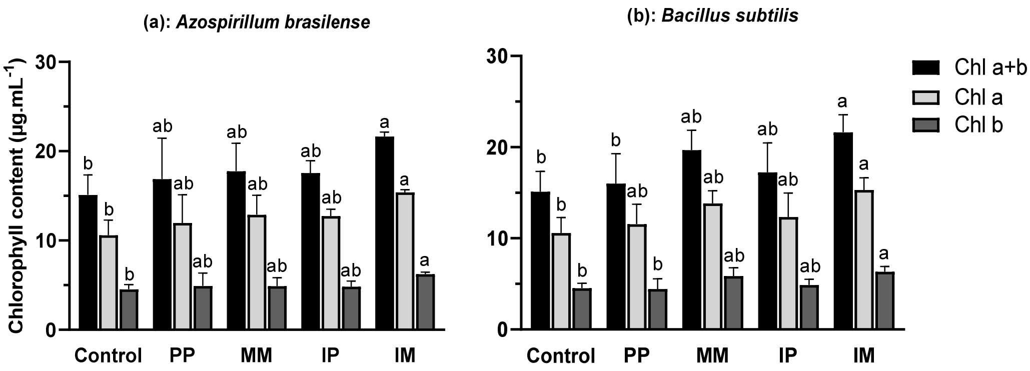

Figure 2. Effect of different inoculation methods on chlorophyll levels. Plants were inoculated by (**a**) *Azospirillum brasilense* or (**b**) *Bacillus subtilis*. Values represent the means ± standard deviation (n = 15). Different letters represent significant differences between treatments using one-way ANOVA with Fisher's LSD at p = 0.05.

4. Discussion

PGPRs have a positive effect on improving plant growth and protecting the environment by reducing the use of chemical fertilizers [1]. *Azospirillum brasilense* and *Bacillus subtilis* are both widely recognized bacterial species that are used as plant growth-promoting rhizobacteria (PGPR). *A. brasilense* occurs in the rhizosphere of grasses and cereals, and it has been shown to have a number of beneficial effects on the growth and development of plants, including the production of phytohormones, improving the availability and absorption of nutrients, enhancing plant tolerance to drought and inducing of systemic resistance [16,17]. Similarly, *bacillus subtilis* is also known for its effects in biofertilization and biocontrol [18].

There are various methods used to inoculate PGPR in plants. In order to optimize and determine the best way to inoculate the plants to obtain significant results, we used four inoculation methods; inoculation of wheat with PGPR affected wheat growth showed significant beneficial effects on various morphological or physiological responses of wheat. The results showed that pellet inoculation (PP) in plants inoculated with *Azospirillum brasilense* is the inoculation that gives the best results for fresh weight, dry weight, and leaf length. However, inoculating using the (IM) method gave the best results in leaf number and chlorophyll content. Our experiment showed that inoculating plants with *Bacillus subtilis* using the inoculation method (IM) resulted in significant improvements in the growth stage acceleration, leaf length, and chlorophyll content. However, using pellet inoculation (PP) gave the best results for the dry weight of both leaves and roots.

5. Conclusions

The (PP) and (IM) inoculation methods gave the best results. The results varied depending on the strain utilized or the physiological parameter studied.

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