

Enhancement of *Medicago sativa* Plant Growth and Yield after Treatment with *Rhizobium* spp.

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ABSTRACT

Rhizobium plays an important role in cultivation of legumes throughout the nitrogen fixation process. In the present study, the growth of alfalfa (*Medicago sativa*) was studied after treatment with *Rhizobium* spp. isolated from two different sites in Jordan. The effect of the two isolates (RS1 and RS2) and co-inoculation of the two isolates (RS1+RS2) was tested on alfalfa. Chlorophyll contents, relative water content, leaf area, shoot length, stem diameter and plant biomass (leaf, root and shoot dry weight) were studied under growth chamber conditions (day/night temperatures of 25°C; 16:8 h photoperiod) using different inoculum doses (10 ml, 20 ml and 40 ml). The effect of *Rhizobium* isolates on alfalfa was compared with rhizosphere plant growth-promoting rhizobacteria (PGPR) collected from the same soil samples. The results indicated that RS1 at high dose (40 ml) and RS2 at any dose significantly enhanced alfalfa growth in all measured parameters compared with control plants (without bacterial inoculation). RS2 was better than RS1 in most measured parameters even at low inoculum dose (10 ml), whereas no significant differences between the two strains were found at high inoculum doses. Co-inoculation of the two isolates was less effective than each isolate alone. On the other hand, the PGPR showed a significant enhancement of alfalfa growth as they enhanced significantly all measured parameters compared to the control plants at all doses.

Keywords: alfalfa, plant growth parameters, *Rhizobium* spp., PGPR.

INTRODUCTION

Year after year, the agricultural sector develops new methods or technologies to increase production yield. Fertilizers play a vital role in boosting yields, but in developing countries, including Jordan, cost is seen as a major hurdle. Recently, the agriculture sector centralized on the use of bio-fertilizers to solve these problems (Zambrano-Mendoza et al., 2021). Bio-fertilizers are substances that contain living microorganisms, specifically bacteria that enhance plant growth and increase yield (Pereg and McMillan, 2014). These beneficial bacteria are called plant growth promoting Rhizobacteria (PGPR). They create a symbiotic relationship with plant roots to improve the growth and productivity of several

crops including canola, wheat, rice, and legumes (Bhattacharya et al. 2013). This relationship occurs by several mechanisms, which include nitrogen-fixing bacteria (Diazotrophes), phosphate solubilizing bacteria, phytoestrogens (microbes expressing phytohormones such as *Azospirillum* that promote plant growth by producing plant hormones), and biological control agents such as *Pseudomonas* spp. and *Bacillus* spp. that protect plants against phytopathogenic organisms (Trabelsi and Mhamdi 2013).

PGPR enhance the growth of different plants such as barley via facilitation of phosphate uptake from soil (Barber et al. 1976), sorghum, peanut, oats, cucumber, cotton, and sunflower via increasing the availability of iron in the root zone (Jones, 2020), as well as enhancement of chickpea

growth and germination under saline conditions (Mishra et al. 2010). Furthermore, they colonize vetch roots and enhance their growth through a wide variety of mechanisms (Yolcui, 2012). Rhizobium symbiosis with legume species is a very important process, producing 50% of the 175 million tons of total biological-N₂ fixation annually worldwide (Ogulcu et al., 2010). *Rhizobium* spp. converts N₂ to ammonia by nitrogenase enzyme, which is used by plants for the synthesis of protein, DNA, RNA, chlorophyll, auxins, cytokinins, alkaloids and glucosinolates (Younesi et al. 2013).

Alfalfa (*Medicago sativa*) is one of the most widely grown legume worldwide. Alfalfa is a perennial legume, that is characterized by short life cycle, self-fertility, high natural diversity, high nutritional quality, high protein content, and easy to create symbiotic relationship with *Rhizobium* spp. for nitrogen fixation to enhance their growth (Kassaw and Frugoli 2012). In Jordan, the local production rate of various forages (Barley, corn, hay, bran, vetches, and alfalfa) was estimated to be about 268 thousand tons compared to 1.3 million tons imported annually to cover the animal feeds for dairy production (Lafi 1995; Alqaisi et al. 2009). The planted areas of alfalfa decreased in the past 14 years due to their poor yield, which occur due to the use of the traditional methods in production and the low productive lands used by farmers, because they use the more productive lands for wheat production (Hadad and Snobar 2011).

Thus, it is necessary to enhance alfalfa growth by providing suitable conditions and nutritional requirement. As such, many researchers indicated the importance of *Rhizobium* inoculants in increasing plants growth and their yield in different soil types and location. Therefore, this study attempted to evaluate the effect of locally isolated *Rhizobium* species in enhancement of alfalfa growth and yield under different treatment conditions.

MATERIALS AND METHODS

Soil samples

Soil samples were collected from alfalfa crops rhizosphere from two different locations: Al-Sokhnah 25 km Northeast of Al-Zarqa city, and from Al-Azraq region, 80 km East of Al-Zarqa city. The rhizosphere soil and the root samples (from alfalfa crops) were taken at a depth of 0–15

cm (3 samples/location), kept in plastic bags then transferred to the laboratory.

Isolation and identification of *Rhizobium* species

Ten grams of soil were added into 90 ml of sterile distilled water. The soil suspension was diluted with sterile distilled water to prepare serial dilution from 10⁻¹ to 10⁻⁵ concentration. One ml suspension was poured into YEM agar plates having the following composition per liter: K₂HPO₄ 0.5 g, MgSO₄·7H₂O 0.2 g, NaCl 0.1 g, mannitol 10 g, yeast extract 1 g, agar 15 g (Dubey 2000; Ogulcu et al. 2010; Younesi et al. 2013; Shahzad et al. 2012; Cogorcena et al. 1997). The plates were incubated at 28°C for 48 hours. Thereafter, bacterial colonies which appear gummy, translucent or white opaque were picked up and streaked on a second YEM agar plates as a subculture. A single colony was transferred on YEM agar slant for preservation (Dubey and Maheshwari 2004).

The isolates were identified by investigating: (i) morphological characteristics that include colony morphology (color, mucosity, borders, transparency, and elevation), and Gram-stain reaction; (ii) biochemical tests which include absorption of congo red in congo red yeast extract mannitol test (CRYEMA), acidic/alkaline pH using YEM containing bromothymol blue as an indicator as described by Dubey (2000), catalase test, oxidase Test, gelatine liquefaction test, citrate utilization test, starch hydrolysis test, urea hydrolysis test, methyl red test, voges-proskauer (VP) test, indol test, triple sugar iron test (TSI) and motility test as described by Dubey and Maheshwari (2004).

Isolation of local rhizobacteria (PGPR)

Ten grams of soil were mixed with 90 ml of sterile distilled water and agitated by Vortex. The soil suspension was diluted with sterile distilled water to prepare serial dilution from 10⁻¹ to 10⁻⁵ concentration. One ml suspension was poured into 99 ml of nutrient broth, incubated for 24 h at 25°C in incubator shaker (Human Lab, Korea) at 125 rpm. After incubation was completed, the broth was used as a stock culture to be used as pots inoculums by (Dubey and Maheshwari 2004).

Effect of *Rhizobium* on alfalfa growth

Cultivation of alfalfa

Alfalfa (*Medicago sativa*) seeds were planted in pots containing sterilized peat moss and perlite mixture (3:1, v: v). The experiment was conducted in growth chamber (Akyurt, Turkey) with day/night temperatures of 25°C and a photoperiod of 16:8 hr (Ogulcu et al. 2010).

Inoculum and inoculation

Rhizobium isolates (RS1, RS2) and PGPR were cultured in YEM broth in a 250 ml flask for 48h at 25°C in incubator shaker at 125 rpm. The OD₆₀₀ for each culture was adjusted to 0.5 (Ogulcu et al. 2010; Younesi et al. 2013). For co-inoculation of both isolates (RS1+RS2) treatment, broth containing *Rhizobium* (RS1) isolated from the Al-Sokhnah region was added to the same volume of the *Rhizobium* (RS2) isolated from Al-Azraq region. The mixture was allowed to stand for 30 minutes at room temperature without shaking, and then used in plant treatment (Younesi et al. 2013). Three different inoculum doses (10 ml, 20 ml and 40 ml) from each bacterial broth were applied by pipette to the rooting medium at the base of the plant.

Experimental design and data collection

The experiment had two factors with 5 replicates and 13 treatment levels in randomized block design. The treatment levels as follows (1) Untreated plants, no *Rhizobium* isolates nor PGPR were applied. (2) *Rhizobium* (RS1)-treated. (3) *Rhizobium* (RS2)-treated. (4) Co-Inoculation *Rhizobium* strains (RS1+RS2)-treated. (5) PGPR-treated. All these treatments were applied at three different volumes (10 ml, 20 ml and 40 ml), as mentioned previously. The plants were left to grow under growth chamber conditions and irrigated with tap water whenever needed.

Every month and for three months from bacterial post treatment, the chlorophyll contents in leaves were measured using chlorophyll index meter (Opti-sciences, USA). To measure the relative water content, one leaf was sampled from one plant per pot. Immediately after cutting their blade, the leaves were placed in a plastic bag and their fresh weight was measured. Turgid weight was determined as the leaves were held in distilled water at room temperature (approximately 20°C) for

16–18 h; then, they were quickly and carefully dried and their turgid weight was determined. Thereafter, the leaves were oven dried for 6 h at 85°C and their dry weight was determined (Rahimia et al. 2010). Finally, their relative water content was calculated according to the following equation:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100 \quad (1)$$

where: RWC, FW, DW and TW – relative water content, fresh weight, dry weight and turgid weight, respectively.

At harvesting time (3 months' post treatment), leaf area using leaf area was measured by using a leaf area meter, stem diameter was recorded by using a digital caliper, and shoot length as well. Consequently, the plants were harvested from the pots and their roots were gently cleaned from soil residues to estimate the number of nodules. Thereafter, the plant was cut into two parts, the root and the above ground shoot. Plant parts were backed separately in paper bags, oven dried for 72 h at 65°C to calculate dry weight of leaf, shoot and root parts for each plant.

Enumeration of bacteria and fungi from plant pots

One gram of soil was taken from pots (2 cm depth) of each treatment and added into 9 ml of sterile distilled water. The soil suspension was diluted with sterile distilled water to prepare serial dilution from 10⁻¹ to 10⁻⁵ concentration, one ml suspension was poured into 19 ml of melted nutrient agar tube (cooled to 45°C) for bacteria and potato dextrose agar (PDA) for fungi. The melted agar was poured in petri plates. The nutrient agar plates were incubated at 37°C for 24 hours and the PDA plates were incubated at 25°C for 4 days (Dubey and Maheshwari 2004). Accordingly, colony forming units (CFU) for bacteria and fungi was recorded.

Statistical analysis

One-way repeated measure ANOVA was used to test the significant differences in the measured variables of treatments including plant biomass, chlorophyll contents, relative water content, leaf area, shoot length and stem diameter using SPSS software. The least significant differences test (LSD) was applied to make comparisons among the means at the 0.05 level.

RESULTS

Isolation and identification of *Rhizobium* species

Out of 20 isolates, two isolates were suspected to be *Rhizobium*, as they were Gram negative rods shaped and have circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high viscosity. The two isolates appeared to be surrounded by yellow color in the YEM agar medium plate with bromothymol blue indicator. They formed white colony color in Congo red YEMA test. The isolates were found to be positive for motility test, catalase and oxidase tests. On the other hand, they showed negative reaction for gelatin liquefaction test, starch hydrolysis test, urea hydrolysis test, methyl red test, voges-proskauer Test (VP), indol test, and hydrogen sulfide production test (H_2S) (Table 1).

For triple sugar iron test (TSI) test, the RS1 isolate produced yellow color slant and yellow color butt of the test tube media without bubbles or black precipitate was observed. In turn, the RS2 isolate did not produce any changes in the test tube media. The two isolates showed different results for citrate utilization test, as the RS1 showed positive result and RS2 showed negative one (Table 1).

Effect of *Rhizobium* isolates on alfalfa growth

Chlorophyll content

After 60 days of planting, plants treated with RS2 at low dose inoculum (10 ml) had a significantly higher chlorophyll content ($\mu\text{g cm}^{-2}$ tissue) compared to the other doses and the un-inoculated control plants. The *Rhizobacteria* isolated from the same soil samples also showed a very strong effect in increasing chlorophyll content at a high dose (40 ml) compared with the other doses, and control plants (Figure 1b).

The plants treated with RS1 significantly increased the chlorophyll content compared to the un-inoculated control plant, and no significant differences occurred between their three doses. However, plant growth rate was less significant than RS2 at low dose and *Rhizobacteria* at high dose (Figure 1a). Co-inoculation of (RS1+RS2) treatment significantly increased the chlorophyll content at a low dose compared to

Table 1. Morphological and biochemical tests for the two *Rhizobium* isolates

Tests	<i>Rhizobium</i> isolates	
	RS1	RS2
Gram stain	-ve	-ve
Colony morphology	circular	circular
Colony color	creamy	creamy
Mucosity	+	+
Bromothymol blue with Medium colony color	yellow	yellow
Congo red with medium Colony color	white	white
Motility	+	+
Oxidase test	+	+
Catalase test	+	+
Gelatine liquefaction test	-	-
Starch hydrolysis test	-	-
Urea hydrolysis test,	+	+
Methyl red test	-	-
Voges-Proskauer (VP)	-	-
Indol test	-	-
Citrate utilization test	+	-
TSI	A/A	K/K

a high dose and control plants, while their significant effect was lower than RS2 at low dose and *Rhizobacteria* at the high dose (Figure 2). In general, RS2 (low dose) and *Rhizobacteria* (high dose) were the best compared to the rest of treatments in enhancing the alfalfa growth, while the weakest of all was the co-inoculation of (RS1+RS2) treatment (Figure 2a).

After 60 days of planting, the results were similar to those obtained during the first period of the experiment (Figure 1b). The RS2 treatment significantly increased chlorophyll content at low and medium doses than at high dose compared to control. In turn, the chlorophyll content was significantly increased in the plants treated with *Rhizobacteria* at all doses. The RS1 treatment increased the chlorophyll content significantly at medium and high doses compared to low dose and control plant. Co-inoculation of (RS1+RS2) significantly increased the chlorophyll content using low dose compared to high dose and control plants, while their enhancement effect was less significant than RS2 and *Rhizobacteria* at (10ml) and RS1 and *Rhizobacteria* at (40 ml) (Figure 3). In general, co-inoculation of (RS1+RS2) was noted to give the least alfalfa growth significance.

After 90 days of the planting plants treated with RS2 at (10 ml) possessed higher chlorophyll content compared to high dose treatment, while RS1 increased the chlorophyll content at (40 ml) dose. Moreover, *Rhizobacteria* increased chlorophyll content more than control with no differences among the other three doses. Co-inoculation of (RS1+RS2) increased the chlorophyll content at low dose compared to the control, but at medium and high doses, no differences were observed (Figure 1c).

Relative water content, leaf area, shoot length and stem diameter

The alfalfa treated with different doses of *Rhizobium* isolates and *Rhizobacteria* showed positive effects on plant RWC, leaf area, shoot length and stem diameter (Figure 2). Results showed that a low dose (10 ml) of RS2 increased plant RWC and leaf area more than the rest of doses, as well as the control (Figures 2a and 2b). Regarding stem diameter and shoot length, differences occurred only between low and high doses. The plants treated with low dose of RS2 showed thicker stem diameter and taller shoot compared to high dose and control (Figures 2c and 2d). The plants

treated with *Rhizobacteria* showed similar effect as RS2 (significant increase in RWC, leaf area, shoot length and stem diameter compared to the control). High dose of *Rhizobacteria* was the best in promoting RWC and leaf area over the other doses (Figures 2a and 2d).

RS1 showed no differences in RWC between their doses and in comparison with the control (Figure 2). Nevertheless, they increased leaf area over the control but no differences between their doses were noted (Figure 2a). RS1 resulted in taller shoots and thicker stems at high dose compared to other doses and the control (Figures 2c and 2d). Leaf area increased in the plants treated with co-inoculation of (RS1+RS2) at low dose compared to the control. No differences occurred between other doses and control when the dose was increased. The plants treated with co-inoculation of (RS1+RS2) bacteria showed a similar effect as control in RWC, shoot length and stem diameter and lower than other treatments (Figure 2).

Root, shoot and leaf dry weights

Results showed that RS2 increased the dry weight of leaf, root and the shoot at a low dose compared to the control, while higher

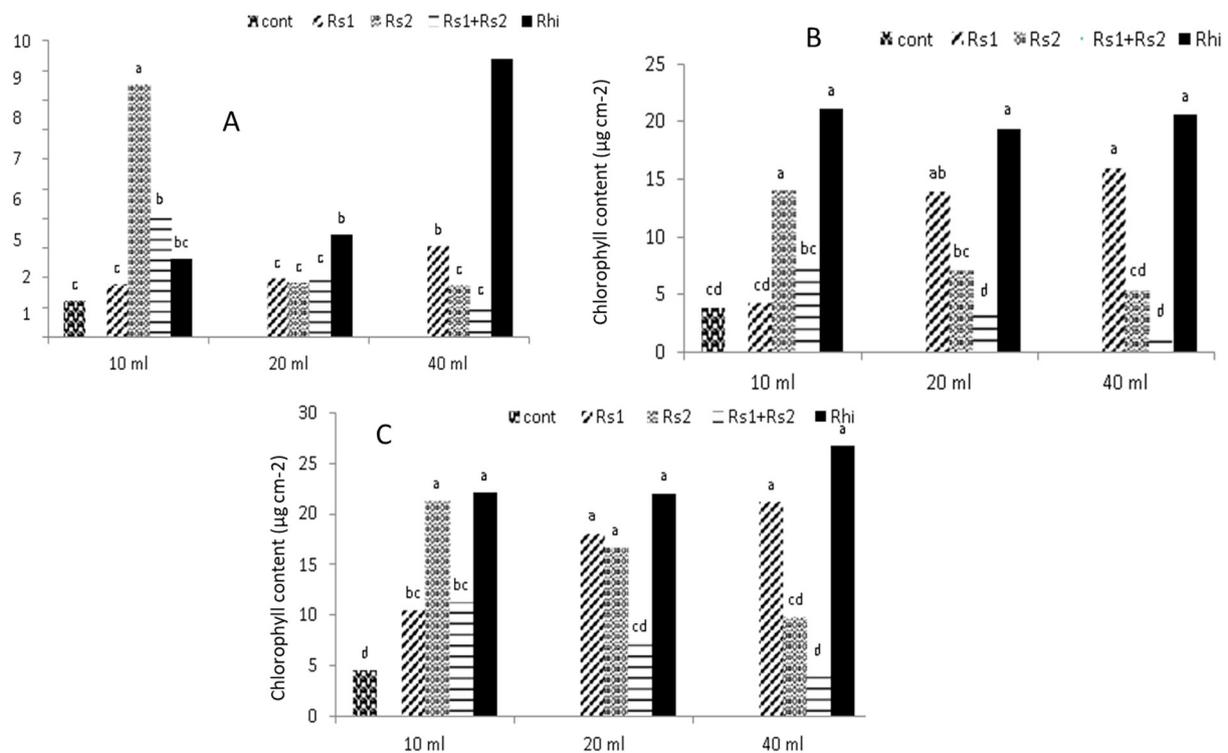


Figure 1. Effect of *Rhizobium* strains and PGPR on the chlorophyll content of the alfalfa leaves of 30-days (a), 60-days (b) and 90-days (c)- post treatment under varying levels of each bacterium. Mean value with the same letter are not significantly different at $P = 0.05$ according to LSD test

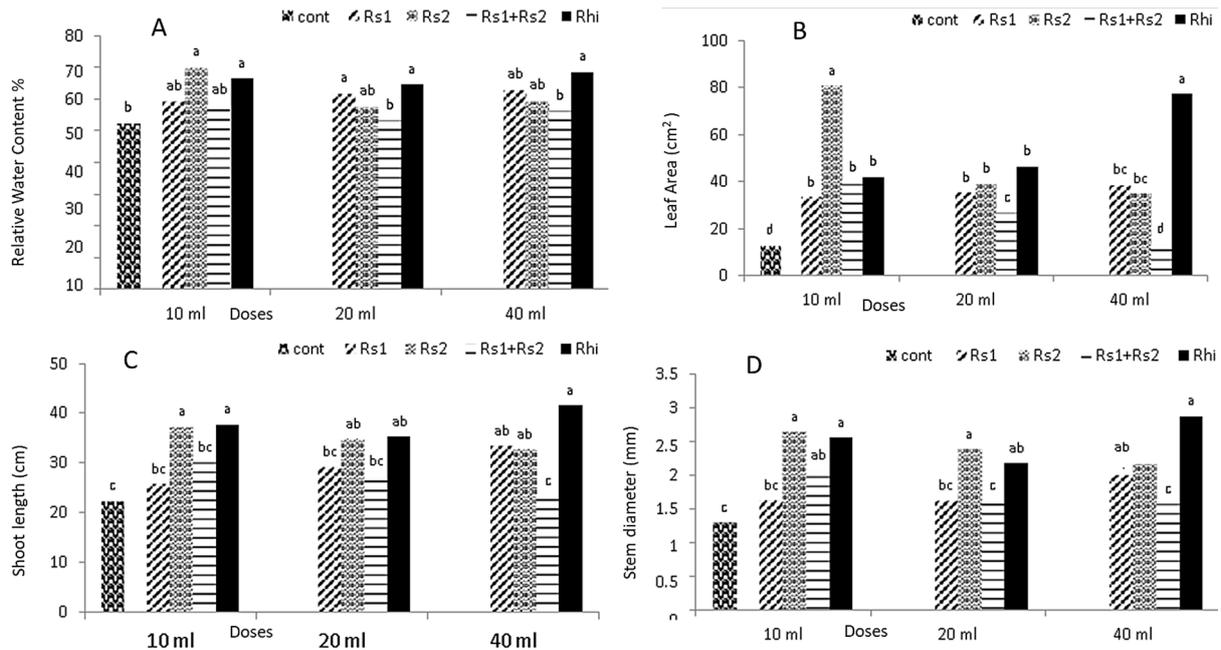


Figure 2. Effect of *Rhizobium* strains and PGPR on relative water content of the alfalfa leaves, (a), leaf area (b), shoot length (c), and stem diameter (d) of 90-days-post treatment under varying levels of each bacterium. Mean value with the same letter are not significantly different at P = 0.05 according to LSD test

doses of RS2 reduced the dry weight of the plant same as the control (Figures 3). The plants treated with Rhizobacteria showed strong significant differences in leaf dry weight among low and high doses. The increased leaf dry weight was observed at the high dose (Figure 3a). *Rhizobacteria* at all different doses

increased roots dry weight at relatively same rate, while control had no effect (Figure 3b). The plants treated with high dose of *Rhizobacteria* possessed a heavier shoots compared to the other three doses and control (Figure 3c). The RS1 treatments resulted in positive significant differences in leaf dry weight compared to the

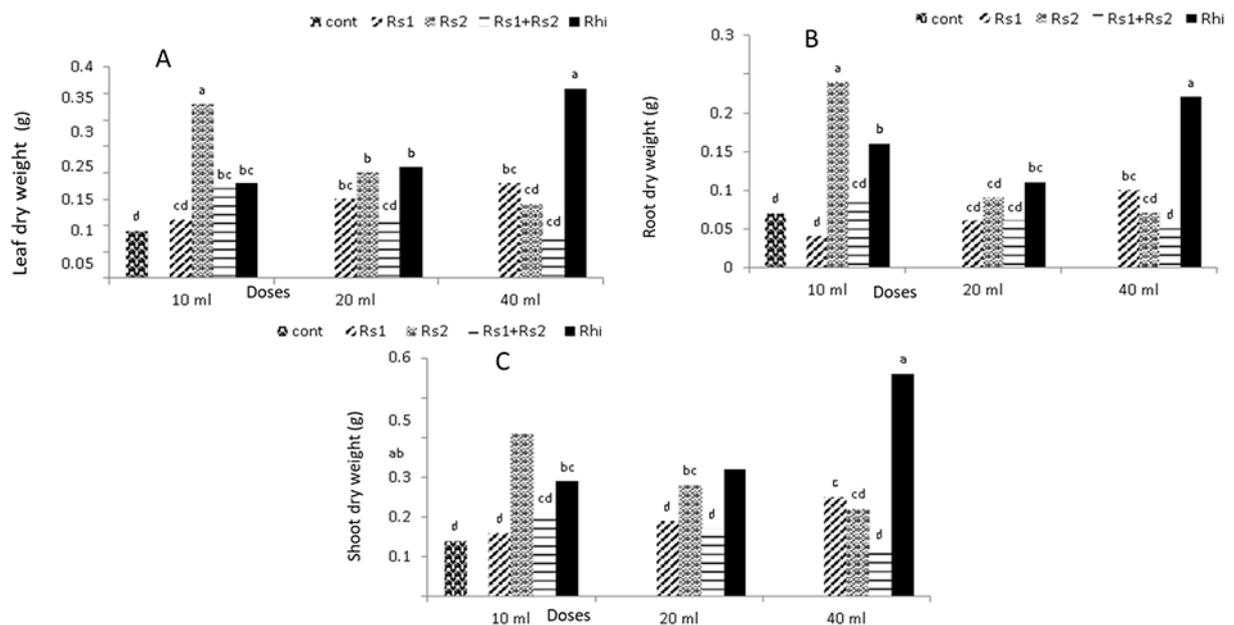


Figure 3. Effect of *R* varying rhizobium strains and PGPR on alfalfa leaf dry weight (a), root dry weight (b), and shoot dry weight (c) of 90-days post treatment under levels of each bacterium. Mean value with the same letter are not significantly different at P = 0.05 according to LSD test.

control, but not to other doses. The three doses of RS1 did not increase roots dry weight taking note that high dose of RS1 increased shoots dry weight compared to the other doses and control (Figure 3c). The co-inoculation of (RS1+RS2) increased leaf dry weight in comparison to the control, and no differences occurred among its three doses (Figure 3).

Number of nodules

The results showed that the plants inoculated with low dose of RS2 and high dose of *Rhizobacteria* produced more nodules (Table 2). In contrast, the plants inoculated with high dose of co-inoculation of (RS1+RS2) bacteria were the lowest.

In general, as it was obviously noticed, after 90-days post treatment showed a clear enhancement of alfalfa growth treated with RS2 compared to control. RS2 at low dose was better than the other doses. The plant treated with high dose of RS1 was better than low dose and the control plant. *Rhizobacteria* showed a very strong effect in enhancing alfalfa growth at the three doses in comparison to the control, but at the high dose it was better than the other doses. Co-inoculation of (RS1+RS2) treatment at low dose showed the best result than the others including the control. Thus, the order of bacteria according to their enhancing effect on alfalfa growth was as follows: RS2 and *Rhizobacteria* > RS1 > co-inoculation of (RS1+RS2) > control.

DISCUSSION

Modern agriculture has increasingly focused on the use of microbial products as alternatives to chemical fertilizers. The benefits from this replacement include lower costs for farmers, less pollution and fewer side effects on human health. Biofertilizers will be the best solution to replace chemical fertilizers, which contains mainly active *Rhizobium* strains that play a critical role in enhancing plants growth and yield through nitrogen fixation mechanism (Fahde et al., 2023).

In this study, the authors successfully isolated 2 *Rhizobium* spp. from soil. These bacteria were isolated using YEMA medium and identified according to morphological and biochemical analysis. The isolates were Gram negative, rod shaped, forming circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high production of mucus. According to Dubey (2000), Ogulcu et al. (2010), and Gachande and Khansole (2011), the isolates were classified as *Rhizobium* spp. Both isolates showed differences using triple sugar iron test (TSI) where the RS1 produced yellow color slant and butt (A/A) without bubbles or black precipitate production, due to Glucose, Lactose and Sucrose fermentation with no Gas or (H_2S) production which is in agreement with Shahzad et al. (2012). In turn, RS2 produced no changes in test tube media (K/K). The two strains also showed different results for citrate utilization as RS1 gave positive result while, RS2 gave negative

Table 2. Total number of nodules formed for each treatment and the number of microbial community as represented by *Rhizobacteria* and fungi for each treatment

Dose	Treatments	Total nodules number	Bacteria number (cfu ml ⁻¹)	Fungi number (cfu ml ⁻¹)
	Control (T1)	0	72 x 10 ⁴	18 x 10 ⁴
Low dose (10 ml)	RS1 (T2)	18	15 x 10 ⁴	25 x 10 ⁴
	RS2 (T3)	37	5 x 10 ⁴	7 x 10 ⁴
	RS1+RS2 (T4)	15	20 x 10 ⁴	4 x 10 ⁴
	Rhi (T5)	23	39 x 10 ⁴	12 x 10 ⁴
Mediumdose (20 ml)	RS1 (T6)	20	20 x 10 ⁴	20 x 10 ⁴
	RS2 (T7)	27	30 x 10 ⁴	27 x 10 ⁴
	RS1+RS2 (T8)	12	72 x 10 ⁴	10 x 10 ⁴
	Rhi (T9)	20	42 x 10 ⁴	6 x 10 ⁴
High dose (40 ml)	RS1 (T10)	18	8 x 10 ⁴	12 x 10 ⁴
	RS2 (T11)	16	47 x 10 ⁴	37 x 10 ⁴
	RS1+RS2 (T12)	0	93 x 10 ⁴	11 x 10 ⁴
	Rhi (T13)	35	29 x 10 ⁵	7 x 10 ⁴

one. Gachande and Khansole (2011) isolated a *Rhizobium* spp. that was citrate utilizer. The two isolates showed some common features, as their colonies were surrounded by yellow color when grown in YEM agar medium with bromothymol blue as indicator due to their organic acid production. They formed white colony color in CRYEMA test due to a very slow absorption of Congo red dye (Ogulcu et al. 2010).

In the experiments employing both isolates to study their effect on alfalfa growth, the results indicated that *Rhizobium* isolates significantly increased plant growth compared with the control plant (without bacterial inoculation) in all measured parameters (chlorophyll contents, RWC, leaf area, plant biomass, shoot length and stem diameter) due to their role in symbiotic interaction with legumes for nitrogen fixation in root nodules (Mohammadi and Sohrabi 2012; Zhao et al. 2012). Many authors have established that chlorophyll synthesis is dependent upon mineral nutrition. Mineral nutrition significantly affects the leaf surface formation and the extent of leaf surface, which is reflected in the sum total of leaf surface, the photosynthetic potential, and pure productivity of photosynthesis (Muraleedharan et al. 2010). Of all macro-elements, the greatest influence on the development of plants in general and their leaf surface is nitrogen. Nitrogen concentration in green vegetation is related to chlorophyll content, and therefore indirectly to one of the basic plant physiological processes: photosynthesis. Nitrogen supply has large effect on leaf growth because it increases the leaf area of plants and, on in this way, it influences photosynthesis (Bassi et al. (2018).

Several researchers reported *Rhizobium* role in promoting alfalfa growth (Ramachandran et al. 2011; Thamer et al. 2011) and other plants as lentil, chickpea, soy bean etc. (Wong 1980; Outcu et al. 2008; Ogulcu et al. 2010; Rajpoot and Panwar 2013; Kasim et al. 2016). Other researchers referred to *Rhizobium* role in plant growth enhancement while inoculation with PGPR and co-inoculation with *Rhizobium* bacteria as the case of this study (Martins et al. 2004; Mirza et al. 2007; Terpolilli et al. 2008; Fox et al. 2011; Stajovic et al. 2011; Glick 2012; Muhammad Aamir et al., 2013)

They all emphasized the role of PGPR and *Rhizobium* sp. in enhancing many traits other than nitrogen fixation such as IAA production, phosphate solubilization and siderophores production that all lead to improve plant growth. RS2 at low dose was more effective than RS1 in enhancement

of alfalfa growth. This is supported by nodules number, chlorophyll content, plant biomass, and in other measured parameters. This effectiveness was due to symbiotic interactions. Nitrogen fixing symbiosis can vary from those that fix little or no nitrogen to those that fix at level equivalent to or even greater than plant requirement (Dashadi et al. 2011; Kasim et al. 2016).

Significant differences in promoting the effect of alfalfa growth between the two isolates at low dose could be related to the presence of fungi in soil pots (Table 2). The number of fungi in the soil pots that was treated with RS1 and RS2 was 25×10^4 and 7×10^4 respectively. Various studies referred to fungi role in reducing the nodulation and nitrogen fixation in legumes (Mirza et al. 2007). Furthermore, fungi produce mycotoxins that are responsible for declining *Rhizobium* strain effect. However, this effect is variable, depending on *Rhizobium* and strains, types of host legumes and the types of fungi involved (Goyal and Habtewold, 2023) and (Habte and Barrion 1984).

RS2 at low dose significantly increased alfalfa growth in all measured parameters compared to their high dose. Increased RS2 doses led to a decrease in stimulating plant growth. This might be related to increased competition at high dose between the strain and the microbial community in their pot soil. The pot soil of RS2 at high dose possessed a higher microbial community number compared to the other doses. The number of microbial community in soil pots that were treated with RS2 was 47×10^4 (Table 2). Thus, RS2 can be suspected to have a low competitive capability. Although it is highly effective in enhancing plant growth, some researchers suggested that no relationship was detected between strain effectiveness and competitive ability (Gottfred and Christle 1989). Other studies have shown that effective strains are not necessarily more competitive than ineffective strains (Ireland and Vincent, 1988). The successful establishment of an introduced strain in soil containing an indigenous population has been attributed to the concentration of inoculums, strain effectiveness, soil factors, host genotype and competition with other rhizosphere organisms (Naeem et al., 2004, Park et al., 2023).

To form the majority of nodules an introduced strain must be more competitive than being indigenous or competing strain, although effectiveness and competitiveness are not related, successful inoculant strains must possess both traits (Naeem et

al. 2004, Andersen and Poole, 2021). RS1 at high dose significantly increased the alfalfa growth compared with control and other doses. RS1 increased plant growth when its dose was elevated. This might be related to the plant need of a high concentration of this strain to obtain large amounts of nitrogen fixation and high nodules number. Amarger and Lobreau (1982) and Swarnalakshmi et al. (2020) claimed that the increased number of bacteria in the inoculums enhances the number of nodules, which in turn is reflected on the rate of plant growth.

No significant differences were recorded between RS1 and RS2 at high doses in stimulating plant growth. As mentioned before, this might be due to the high number of microbial community in the soil pot of alfalfa treated with RS2 and the increased competitiveness compared with RS1 (Table 2). The number of fungi in the soil pot of the plants treated with RS2 was 37×10^4 , 3 folds higher than in the case of RS1 (12×10^4), which may reduce the RS2 effect. The effect at high dose indicated that RS1 was less effective than RS2.

Co-inoculation of RS1+RS2 at low dose significantly increased chlorophyll content, stem diameter, leaf area and leaf dry weight (Figure 1, 2, and 3) compared with the control, but without significant differences at all of their doses on shoot length, shoot dry weight, RWC, root dry weight (Figure 2 and 3). Furthermore, no significant differences occurred when co-culturing both isolates at high dose. This indicated the weakness of the co-inoculation of (RS1+RS2) treatment compared to each strain alone in enhancing plant growth, which might be related to competitiveness between the two strains.

CONCLUSIONS

In the light of the present results discussed in details above, it can be concluded that *Rhizobium* isolates collected from two different locations in Jordan positively affected all parameters of growth such as chlorophyll content, RWC, stem diameter, shoot length, leaf area, number of nodules, and dry weight of root, shoot and leaf in alfalfa plant. The best performance was obtained from *Rhizobium* RS2 and PGPR treatments in all measured parameters compared to RS1, co-inoculation and control treatments. RS1 was less effective than RS2. The co-inoculation

of the two isolates was not effective in enhancing plant growth. The experiments were conducted under growth chamber conditions; however, it is important to study the effects of these isolates strains under field conditions.

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