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Delivery of Inoculum of *Rhizophagus irregularis* via Seed Coating in Combination with *Pseudomonas libanensis* for Cowpea Production

Ying Ma [1,*], Aleš Látr [2], Inês Rocha [1], Helena Freitas [1], Miroslav Vosátka [3,4] and Rui S. Oliveira [1,5]

1 Centre for Functional Ecology-Science for People & the Planet, Department of Life Sciences, University of Coimbra, 3000-456 Coimbra, Portugal; ines.sousa.rocha@gmail.com (I.R.); hfreitas@uc.pt (H.F.); rsoliveira@uc.pt (R.S.O.)
2 Symbiom Ltd., 563 01 Lanškroun, Czech Republic; latr@symbiom.cz
3 Institute of Botany, Academy of Sciences of the Czech Republic, 253 43 Průhonice, Czech Republic; miroslav.vosatka@ibot.cas.cz
4 Department of Experimental Plant Biology, Charles University, Faculty of Science, 128 44 Prague, Czech Republic
5 Department of Environmental Health, Research Centre on Health and Environment, School of Allied Health Sciences, Polytechnic Institute of Porto, 4200-072 Porto, Portugal
* Correspondence: cathymaying@gmail.com; Tel.: +351-239-240-752

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Abstract: Cowpea (*Vigna unguiculata* L. Walp) is an important legume grown primarily in semi-arid area. Its production is generally inhibited by various abiotic and biotic stresses. The use of beneficial microorganisms (e.g., plant growth promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF)) can enhance agricultural production, as these microorganisms can improve soil fertility and plant tolerance to environmental stresses, thus enhancing crop yield in an eco-friendly manner. Application of PGPB and AMF in large scale agriculture needs to be improved. Thus, the use of seed coating could be an efficient mechanism for placement of inocula into soils. The aim of this study was to evaluate the effects of the AMF *Rhizophagus irregularis* BEG140 and the PGPB *Pseudomonas libanensis* TR1 alone or in combination on the biomass and physiological traits of cowpea. Four treatments were set: (i) non-inoculated control; (ii) PGPB; (iii) AMF applied via seed coating; and (iv) PGPB + AMF applied via seed coating. Cowpea plants inoculated via seed coating with *R. irregularis* and those inoculated with *R. irregularis* + *P. libanensis* showed root mycorrhizal colonization of 21.7% and 24.2%, respectively. PGPB *P. libanensis* was efficient in enhancing plant biomass and seed yield. There was no benefit of single (AMF) or dual (PGPB + AMF) inoculation on plant growth or seed yield. The application of beneficial soil microorganisms can be a viable approach for sustainable cowpea production in precision agriculture scenarios.

Keywords: seed coating; plant growth promoting bacteria; arbuscular mycorrhizal fungi; *Vigna unguiculata*; sustainable agriculture

1. Introduction

Abuse of agrochemicals in agriculture to meet the needs of a growing human population can deteriorate the overall ecosystem quality and compromise the environment and public health. Sustainable agriculture has focused on developing new practices including the use of beneficial microorganisms as a safe and eco-friendly tool for fostering food production without compromising ecosystems services. Beneficial microbes, such as arbuscular mycorrhizal fungi (AMF) and plant

growth promoting bacteria (PGPB), representing a key functional interface between plant roots and soils, are considered as natural biofertilizers, due to their ability to exert direct and indirect beneficial effects on soil quality and structure, crop growth and quality, abiotic (e.g., drought, salt, metals and extreme temperature) and biotic (e.g., phytopathogens) stress resistance, and consequently agricultural sustainability [1–5].

Cowpea is a very important food source and a widely cultivated legume throughout the world, particularly in Asia, Africa, and Latin America. Cowpea makes great contribution to fulfilling human dietary protein requirements [6]. However, various abiotic and biotic stresses inhibit plant growth and grain yield [7,8]. Thus, the application of beneficial microbes in situ as an elicitor or stimulator could represent a good choice to enhance plant growth under normal or stressful conditions. Plant growth promoting substances produced by PGPB result in reduced use of chemical fertilizers, while the solubilized mineral nutrients (e.g., nitrogen, phosphorus and potassium) and phytohormones (e.g., indole-3-acetic acid and cytokinins) could be available for legume uptake [2,3]. Moreover, legumes are associated with AMF under a complex hyphal network, which improve plant growth and development, mainly through enhancing nutrient uptake [9]. To achieve maximum performance of beneficial inoculum, it is important that the introduced microbes compete effectively against the indigenous microbial community and present their functional traits. Root colonization by AMF is a prerequisite for the success of inoculum application, since it is a critical process in establishment of plant–microbe association [10].

It is noteworthy that the application of beneficial microbes has not yet been widely integrated in agriculture, because of its inadequate inocula delivery process and lack of awareness by farmers. Therefore, the development of a suitable delivery system of beneficial microbes that retains microbial functional activities is crucial for sustainable agriculture. Among common delivery methods, integration of symbiotic microorganisms (e.g., AMF and PGPB) in coating agents around the seed (so-called seed coating) offers great potential to enhance seed establishment and plant field performance in a cost-efficiency way [11,12]. Therefore, this study was conducted to examine the effects of AMF (inoculated via seed coating) and PGPB alone or in combination on the production of cowpea. The specific objectives of the study were: (1) to deliver AMF through seed coating; (2) to evaluate the effects of AMF and PGPB inoculation on the biomass and physiological traits of cowpea; and (3) to determine the colonization capacity of AMF when inoculated via seed coating.

2. Materials and Methods

2.1. Experimental Plant

An important indigenous grain legume, cowpea (*Vigna unguiculata* (L.) Walp. cv. Fradel) was selected for this study, as it has high-protein seeds and nutrient-rich edible leaves, which contribute to fulfilling the high nutritional requirements of humans. In addition, it may tolerate drought and heat, and can thus be grown successfully in many areas.

2.2. Bacterial Strain

The abiotic stress (drought, salt and heavy metals) resistant PGPB strain *Pseudomonas libanensis* TR1 (GenBank accession no. KR051238) originally isolated from the rhizosphere of *Trifolium repens* grown in serpentine soils in Bragança, northeast of Portugal, was obtained from the culture collection of the Centre for Functional Ecology, University of Coimbra [13]. *P. libanensis* TR1 was chosen in this study due to its beneficial biochemical characteristics, as shown in Table 1. *P. libanensis* TR1 exhibited tolerance to heat (38 °C), salinity (8%) and severe drought (−1.5 Mpa). It was able to fix N_2, solubilize P, as well as produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophore, IAA and ammonia (NH_3). Overnight grown bacterial culture was centrifuged at 6000 rpm for 10 min and the pellet was washed twice with biological saline (0.85% KCl). The pellet was resuspended in

biological saline and the optical density (600 nm) of bacterial suspension was adjusted to 1 (about 1×10^9 colony-forming units per mL).

Table 1. Biochemical characteristics of *Pseudomonas libanensis* TR1.

Characteristic	Parameter	Unit	*P. libanensis* TR1
Abiotic stress resistance	Salt tolerance	%	8
	Osmotic tolerance *	nq	+
	Temperature tolerance	°C	4–38
Plant growth promoting traits	ACC deaminase production	μm α-KB mg^{-1} h^{-1} protein	34.2 ± 6.7
	P solubilization	nq	+
	IAA production	mg L^{-1}	88.2 ± 5.6
	Siderophore	CAS: mm	1.0 ± 0.1
	N_2 fixation	nq	+
	NH_3 production	nq	+

* Final water potential of the polyethylene glycol (PEG)-infused plate (−1.5 Mpa); ACC, 1-aminocyclopropane-1-carboxylate; α-KB, α-ketobutyrate; P, phosphate; IAA, indole-3-acetic acid; CAS, chrome azurol S; NH_3, ammonia; N_2, nitrogen; +, positive; nq, not quantified.

2.3. Arbuscular Mycorrhizal Fungal Inoculum and Seed Coating

The AMF isolate *Rhizophagus irregularis* BEG140 provided by Symbiom Ltd., Lanškroun, Czech Republic was multiplied with host plant *Zea mays* L. in a multi-spore pot culture containing a mixture of zeolite and expanded clay (1:1; *v:v*) for six months. The inoculum was sieved through a 250 μm mesh and mixed with the coating material silicon dioxide and starch (1:1:1 *w/w*). The most probable number (MPN) [14] value of the inoculum was ca. 400,000 infective propagules (IPs) per kg. Cowpea seeds were surface-sterilized with 0.5% (*v/v*) sodium hypochlorite for 10 min. After misting with sterile distilled water, seeds were coated by gradually adding inoculum-coating material mixture according to the pan coating method [5,15] and then air dried at 25 °C for 24 h. This resulted in ca. 9 IP per plant and a buildup of 50% of seed weight.

2.4. Microcosm Experiments

For a pot experiment, a sterile substrate mixture composed of sand/loam soil:sand (2:1; *v/v*) was used. Substrate sterilization was done by autoclaving (twice at 121 °C for 1 h, with 24 h delay between the subsequent cycle). Main characteristics of the substrate were 94.3% of dry matter, 945 $mg \cdot kg^{-1}$ of K, 48 $mg \cdot kg^{-1}$ of P, 307 $mg \cdot kg^{-1}$ of N, 4.8 $mg \cdot kg^{-1}$ of S, 37 $mg \cdot kg^{-1}$ of Mg, 870 $mg \cdot kg^{-1}$ of Ca, and pH 7.2.

Pots (height 20 cm, diameter 16 cm, volume 3 L) containing 1 kg of substrate that were arranged on a greenhouse bench in a randomized block design included four treatments: (i) non-inoculated control; (ii) PGPB; (iii) AMF inoculated via seed coating; and (iv) PGPB + AMF inoculated via seed coating. Each treatment was replicated six times (six pots), each pot contained 2 seedlings. Pots of the control and PGPB treatments received two cowpea seeds coated using the same procedure described above except without AMF inoculum. Additionally, pots from the bacterial treatments received 1 mL of the bacterial suspension described above. Non-bacterial treatments received 1 mL of biological saline. Since our study focused on determining host–microbe compatibility and possible growth response, the bacterium was not introduced via seed coating. *Pseudomonas libanesis* showed low shelf life when coated on seed surface (personal communication); therefore, the bacterial suspension was applied directly into the seed vicinity. Pots of AMF coating treatments received two cowpea seeds coated with *R. irregularis* BEG140. Plants were grown in a greenhouse under 16/8 day/night regime for 10 weeks. Temperature and soil water content ranges were 25–30 °C and 55%–70%, respectively. Pots of different treatments were rotated weekly to different bench positions throughout the greenhouse to minimize positional effects.

2.4.1. Plant Biomass

At harvest, plants were carefully washed free of adhering substrate and separated from above-ground biomass (shoots and pods), and then fresh weight of shoots, roots and pods was measured. The number of seeds per pod and seed weight per pod was also recorded. The corresponding dry weight was subsequently determined following oven drying to a constant weight at 85 °C. The shoot to root dry weight ratio, which reflects overall shoot to root balance, was calculated. Aliquots (2 g) of fresh leaves were separated and frozen in liquid nitrogen for determination of pigment content. Roots of all treatments were sampled to determine AMF colonization.

2.4.2. Pigment Estimation

Chlorophyll contents in cowpea leaves were determined by the *N*,*N*-Dimethylformamide (DMF) method [16]. Leaves (0.5 g) were homogenized in chilled DMF and stored at 4 °C in darkness for 16 h. Photosynthetic pigments (chlorophyll a, chlorophyll b and chlorophyll a + b) were estimated spectrophotometrically and calculated using the equations of Lichtenthaler and Wellburn [17].

2.4.3. Mycorrhizal Colonization

Root samples were cut into segments and stained with 0.05% trypan blue in lactoglycerol [18]. Colonization was expressed as percent root length colonized (RLC) by AMF and estimated according to the grid-line intersect method [19] using an ocular grid at 100× magnification.

2.4.4. Bacterial Analysis

At harvest, 1 g of roots and rhizosphere soil was gently collected in 2 mL microtubes. Tubes were maintained at 4 °C and immediately transported to the laboratory for further processing. Once in the laboratory, tubes were frozen (−20 °C) until molecular analyses were carried out. Individually, an aliquot of approximately 0.15–0.2 g was used for DNA extraction using the NucleoSpin® soil extraction kit (Macherey-Nagel), and the obtained DNA was stored at −20 °C. Specific primers targeted at *Pseudomonas* 16S rRNA gene Ps-for (forward primer, 5′ GGTCTGAGAGGATGATCAGT 3′) and Ps-rev (reverse primer, 5′ TTAGCTCCACCTCGCGGC 3′) were used to amplify a specific region (V2 to V8 variable region) of the gene [20]. Amplification was carried out following the conditions described in Rajwar and Sahgal [21], with slight modifications. Reaction mix contained 2.5 µM of each primer, 12.5 µL of DSF Taq Master Mix (BIORON) (2.5 µL of buffer, 200 µM of dNTPs, 0.5 U of DFS-Taq polymerase), and 1 µL of template DNA in a final volume of 25 µL. Amplification was carried out in a T100™ Thermal Cycler (BIO-RAD) and PCR conditions were as follows: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. Five microliters of the obtained PCR products were analyzed using agarose (1%) gel electrophoresis stained with GreenSafe Premium (NZYTech), to verify the presence of the *Pseudomonas* amplicon (990-bp product). After electrophoresis, amplified DNA was visualized using GelDoc™ XR+ system with the Image Lab software (2.0.1) (BIO-RAD).

2.5. Statistical Analysis

The treatment means were compared using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$). All statistical analyses were carried out using SPSS 17.0.

3. Results

3.1. AMF Colonization

The AMF and cowpea formed a symbiotic relationship. *R. irregularis* was able to colonize the roots of cowpea. Inoculation with *R. irregularis* via seed coating resulted in 21.7% and 24.2% RLC in

plants without and with PGPB, respectively (Figure 1). In addition, no AMF colonization was detected in the roots of non-mycorrhizal plants, indicating that there was no fungal cross-contamination among pots of different treatments.

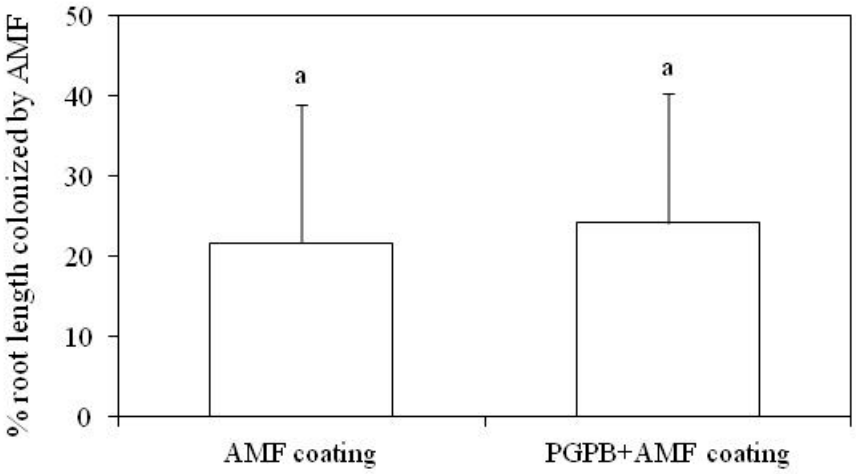

Figure 1. Percentage root length colonized (%RLC) in the roots of cowpea via seed coating. AMF, arbuscular mycorrhizal fungi; PGPB, plant growth promoting bacteria. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$).

3.2. Bacterial Colonization

P. libanensis TR1 was not detected by PCR in the roots and rhizosphere of cowpea plants inoculated with this PGPB strain. Thus, the presence of *P. libanensis* TR1 in our microcosms could not be confirmed after 10 weeks of plant growth.

3.3. Plant Biomass and Chlorophyll Contents

Plants from all treatments had a germination rate of 100%. In our study, the pot experiment was performed under non-stress conditions, which ensured favorable growing conditions. Data indicate that inoculation with AMF *R. irregularis* via seed coating did not affect plant growth (e.g., shoot and root dry weight) (Figure 2). The same trend was observed for plant fresh weight. Notably, *P. libanensis* significantly ($p < 0.05$) increased shoot and total plant dry weight, as well as shoot to root dry weight ratio by 111%, 101% and 83%, respectively, compared to the control treatment. This shows that inoculation of cowpea with PGPB *P. libanensis* was more effective in enhancing plant growth. Nevertheless, dual inoculation with AMF and PGPB inocula resulted in no pronounced changes in plant growth performance in comparison with the control treatment.

Table 2 provides the leaf photosynthetic pigment contents of cowpea. Application of AMF or PGPB alone or in combination did not significantly influence cowpea leaf chlorophyll a, chlorophyll b, chlorophyll a + b, and chlorophyll a/b ratio, compared to the control treatment.

Table 2. Chlorophyll contents in leaves of cowpea.

Treatment	Chlorophyll a (mg g $^{-1}$)	Chlorophyll b (mg g $^{-1}$)	Chlorophyll a + b (mg g $^{-1}$)	Chlorophyll a/b Ratio
Control	1.89 ± 0.43 a	3.04 ± 0.70 a	4.99 ± 1.12 a	0.63 ± 0.06 a
PGPB	2.24 ± 0.22 a	3.50 ± 0.41 a	5.80 ± 0.64 a	0.64 ± 0.02 a
AMF coating	2.21 ± 0.43 a	3.38 ± 0.54 a	5.65 ± 0.95 a	0.65 ± 0.04 a
PGPB + AMF coating	2.33 ± 0.38 a	3.53 ± 0.49 a	5.93 ± 0.88 a	0.66 ± 0.03 a

PGPB, plant growth promoting bacteria; AMF, arbuscular mycorrhizal fungi. Values are means ± standard deviation. Data of columns indexed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$).

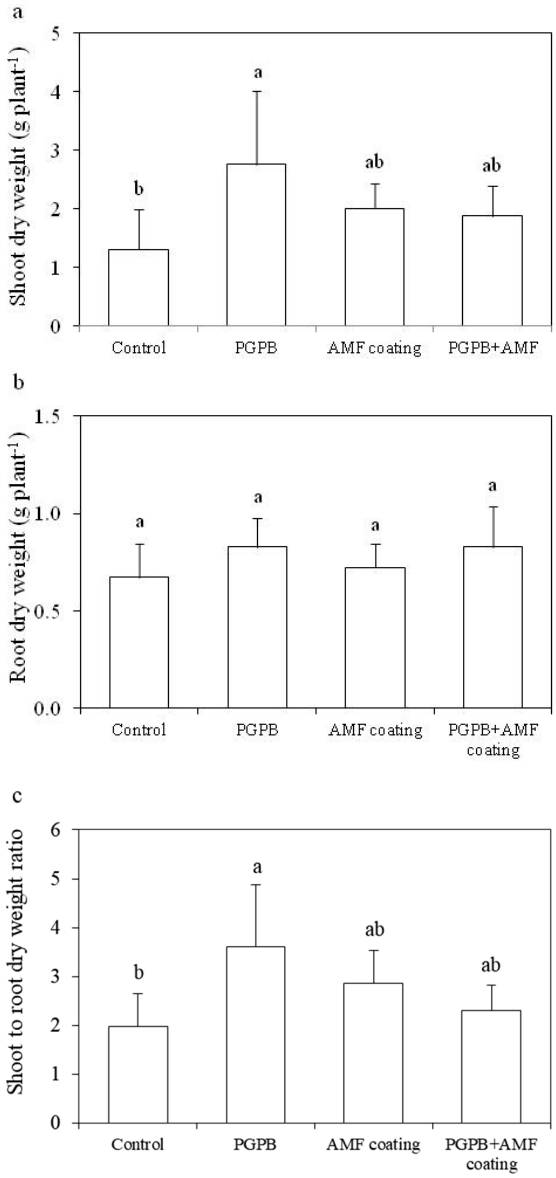

Figure 2. Effects of individual and combined inoculation of PGPB and AMF on shoot (**a**) and root (**b**) dry weight, and shoot to root dry weight ratio (**c**). Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$).

3.4. Effects of Microbes on Seed Yield

The effects of inoculation with *R. irregularis* or *P. libanensis*, alone or in combination on seed production of cowpea were demonstrated in terms of number of pods and seeds, seed weight and seed yield per plant compared with the control (Table 3). AMF inoculation did not ($p > 0.05$) affect seed yield of cowpea, but PGPB inoculation significantly improved the seed yield by 52% when compared to the control treatment. However, co-inoculation with AMF and PGPB resulted in no changes in cowpea seed yield.

Table 3. Number of pods and seeds, seed weight and seed yield of cowpea.

Treatment	Pod Number	Seed Number	Seed Weight	* Seed Yield
	Per Plant	Per Pod	Per Seed (g)	Per Plant (g)
Control	2.3 ± 0.5 a	3.6 ± 0.7 a	0.18 ± 0.0 a	1.43 ± 0.3 b
PGPB	2.6 ± 1.1 a	3.5 ± 0.9 a	0.22 ± 0.0 a	2.18 ± 0.4 a
AMF coating	2.3 ± 0.5 a	2.5 ± 1.5 a	0.18 ± 0.1 a	1.23 ± 0.3 b
PGPB + AMF coating	2.4 ± 0.9 a	3.7 ± 1.8 a	0.17 ± 0.1 a	1.19 ± 0.5 b

Values are means ± standard deviation. Data of columns indexed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$). * Seed yield of a legume plant = number of pods per plant × number of seeds per pod × mean seed weight [22].

4. Discussion

The success of seed coating largely depends on an accurate selection of the coating material, which may influence seed germination [12,23]. In our study, seeds from both non-coated and coated treatments exhibited a germination rate of 100%, showing that the process of seed coating using a mixture of AMF inoculum and silicon dioxide did not reduce the germination. This finding is in accordance with the results of Oliveira et al. [5] on *Triticum aestivum*.

Results of our research revealed successful colonization and survival of AMF *R. irregularis* via seed coating in plants with and without PGPB (Figure 1). Recently, Omirou et al. [24] reported that, in sterilized soils, cowpea inoculated with AMF (a mixed inoculum of *Rhizophagus intraradices* and *Funneliformis mosseae*) and co-inoculated with AMF and a nitrogen-fixing bacterium *Bradyrhizobium* sp. exhibited similar colonization percentages of about 23% and 21%, respectively. The addition of PGPB did not influence the percent RCL compared to AMF-coating treatments without PGPB. Dissimilarly, Krishnamoorthy et al. [25] showed that co-inoculation treatment with the AMF *Rhizophagus intraradices* and bacterium *Massilia* sp. resulted in higher AMF root colonization compared to the *R. intraradices* single inoculation treatment. It is well known that several factors, such as soil structure, organic fertilization, and beneficial bacterial communities, contribute significantly to spore germination, development of fungal mycelia and AMF colonization rate [26–28]. For instance, PGPB may produce metabolites or create a niche that increase spore germination rate and stimulate AMF mycelia in the rhizosphere. The production of plant hormones by PGPB has been suggested as mediating these processes [28]. The enhanced AMF colonization by *Massilia* sp. is probably attributed to the plant growth promoting traits of the bacterial strain and an existing cross talk between the signaling pathways leading to bacterial and mycorrhizal colonization [29]. Our finding displays that application of PGPB was not favorable to the development of *R. irregularis* associated with cowpea. Future research is needed to focus on functional compatibility relationships between AMF and PGPB.

It has been well demonstrated that AMF can alleviate environmental stresses (e.g., drought, salinity and heavy metals) and increase seedling survival rate, growth and development of various plants such as *Allium cepa*, *Calendula officinalis*, *Cicer arietinum*, *Poncirus trifoliata*, *Sesbania sesban* and *Vigna unguiculata* due to their effects on nutrient dynamics and water acquisition [30–36]. However, no significant differences were noted in biomass yield between mycorrhizal and non-mycorrhizal plants (Figure 2). As reported by Muthukumar and Udaiyan [37], soil conditions that favor plant growth can also facilitate the establishment of mycorrhizal association, since they increase the chances for plant roots to encounter AMF. In our study, although well-grown plants (Figure 2) along with successful AMF colonization (Figure 1) were obtained, there were no significant effects of AMF on plant growth. AMF might not benefit plants when net cost of the symbiosis is equal or exceeds net benefit [38]. The absence of growth promotion by *R. irregularis* could also be attributed to the limitations of space for the growth of cowpea in the pots. Thus, the full potential of AMF inocula should be assessed in future studies under field conditions.

The presence of *P. libanensis* TR1 in the roots and rhizosphere of cowpea plants was not detected by PCR. Inoculated PGPB might initially colonize the roots and rhizosphere of target plants at high levels, but these can decline in a few weeks [39]. Despite the decreasing concentration of inoculated

PGPB, their beneficial effect on plant performance can be observed throughout the whole cycle of the target plant [40]. Although our inoculated *P. libanensis* TR1 strain was not detected in the roots and rhizosphere of cowpea after 10 weeks of plant growth, we cannot exclude the positive contribution of inoculated PGPB to the growth and yield of cowpea (Figure 2 and Table 3). PGPB inoculation has been considered as an effective alternative bio-technique to improve plant resistance to environmental stress, quality and yield besides the conventional plant breeding and genetic engineering, which are time consuming and costly [41]. PGPB *P. libanensis* had great potential as a bioinoculant to enhance cowpea growth (e.g., shoot and total plant dry weight). The significant increase in the shoot to root dry weight ratio of PGPB inoculated plants (Figure 2) implies that *P. libanensis* TR1 might also participate in the partitioning of carbon sources and minerals within the plant (shoot-specific activity). The results obtained herein are in agreement with Bashan and Dubrovsky [42]. Moreover, the highest root dry weight, although not significant, was obtained with the treatment of PGPB (Figure 2). It is interesting to note that, in dual inoculation with AMF and PGPB, *R. irregularis* compromised the stimulatory effects of PGPB. This was probably because *R. irregularis* exerted a negative or neutral effect on plant growth within the plant–mycorrhiza–soil system. Similarly, Nouri et al. [43] argued that the negative or neutral growth impact can be attributed to the fact that AMF confer a benefit (e.g., a qualitative benefit) other than growth promotion, or the benefit is not evident under the respective experimental conditions. During symbiotic performance, the role of PGPB on AMF–plant association remains unclear.

The leaf chlorophyll content as an indicator parameter of plant health was determined in all treatments (Table 2). Our findings indicate that, under non-stress conditions, application of PGPB enhanced plant growth performance of cowpea. We assume that PGPB inoculation did not affect chloroplast development in plants; therefore, chlorophyll production maintained no change between non-inoculated and inoculated treatments.

Seed yield has been regarded as an important measure of sustainable crop production. Even though a large range of seed yield has been recorded for cowpea, the production is usually low, due to the soil erosion and low fertility [44]. Our finding displayed that single PGPB inoculation considerably enhanced cowpea seed yield, whereas dual inoculation with AMF and PGPB had no effects (Table 3). Similarly, Valverde et al. [45] found the co-inoculation of phosphate-solubilizing *Pseudomonas jessenii* and N_2-fixing *Mesorhizobium ciceri* strains results in the highest increase in seed yield of *Cicer arietinum*. The positive effects are probably due to a bio-fertilization effect of PGPB [46].

5. Conclusions

The inoculum of AMF applied via seed coating alone or in combination with PGPB successfully colonized the roots of cowpea, demonstrating the success of seed coating as an effective AMF inoculum delivery system. PGPB inoculation significantly enhanced shoot dry weight and total biomass production as well as seed yield of cowpea, which may be attributed to the observed beneficial traits of PGPB *P. libanensis*. The application of beneficial microbes should be encouraged in cowpea growing areas. Our findings indicate that application of AMF via seed coating can serve as an innovative and promising approach for sustainable field-based plant cultivation, as it has the advantage of reducing the amount of inoculum needed. Nevertheless, field scale studies will be essential to validate these finding under real agricultural scenarios.

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