

Article

Seed Coating with Arbuscular Mycorrhizal Fungi for Improved Field Production of Chickpea

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Abstract: Although arbuscular mycorrhizal (AM) fungi are known to promote growth and yield of agricultural crops, inoculation methods for effective scaling up from greenhouse to the field are still underexplored. The application of single or mixed beneficial AM fungal isolates is hindered by the lack of experimental reproducibility of findings at different scales and the cost-effectivity of inoculation methods. Seed coating has been considered a feasible delivery system of AM fungal inocula for agricultural crops. In this study, the impact of single and multiple AM fungal isolates applied via seed coating on chickpea productivity was evaluated under greenhouse and field conditions. Overall, plants inoculated with multiple AM fungal isolates had better performance than those inoculated with single AM isolate under greenhouse and field conditions. While plants in greenhouse displayed higher shoot dry weight (14%) and seed individual weight (21%), in field, inoculation with multiple AM isolates increased pod (160%), and seed (148%) numbers, and grain yield (140%). Under field conditions, mycorrhizal root colonization was significantly higher in chickpea plants inoculated with multiple AM fungal isolates compared to other treatments. These findings highlight the potential of field-inoculation with multiple AM fungal isolates via seed coating as a sustainable agricultural practice for chickpea production.

Keywords: arbuscular mycorrhizal fungi; *Cicer arietinum* L.; field crop production; seed coating

1. Introduction

Chickpea (*Cicer arietinum* L.), one of the main legume crops consumed and cultivated worldwide [1,2], is considered an important and cheap source of nutrients [3] as well as a key crop for soil fertility preservation, especially in rainfed areas [4]. Arbuscular mycorrhizal (AM) fungi are known to promote the growth and yield of legumes, including chickpea [5–8]. According to Rillig [9], the integration of AM fungi in agricultural management strategies is recommended not only for their contribution to crop yield increase, but also for the important roles in ecosystem functions (e.g., soil structure, nutrient conservation, plant stability over changing environment) and potential to reduce the amounts of fertilizer required to achieve cost-effectiveness. AM fungi are capable of increasing the efficiency of agricultural systems through different mechanism such as nutrient uptake regulation, water balances and plant resistance to biotic stresses [10–15]. Additionally, AM fungi can also have a positive and significant influence over grain/seed quality [9,16]. However, factors such as host-plant affinities, soil conditions and the use of single versus multiple AM fungal isolates can

have a great impact on the performance of these beneficial microbes [17,18]. According to Frew [19], agricultural crops can, in general, benefit from higher AM fungal diversity (multiple isolates) in the soil, yet the growth and nutritional advantages depend on the plant-host species. On the other hand, the application of AM fungi in agricultural systems is still restricted due to the lack of cost-effective inoculation methods or the reproducibility of results from greenhouse and field tests [20–22]. In this sense, strategies for developing microbial inoculation methods for broad-scale agricultural production that effectively apply low amounts of inoculants are required.

Seed coating is a process consisting on the application of exogenous materials (including inoculants) onto the seed surface and it has been considered a precise tool with the potential to deliver AM fungi to several agricultural crops, such as wheat, maize, artichoke and cowpea [23–27]. Seed coating ensures the contact of AM fungal propagules with emerging roots assuring colonization at the early plant development stage. Regardless of the potential to increase the productivity and nutrition of different agricultural crops [23,25,28], inoculation of AM fungi via seed coating is still scarce. The scaling up from laboratory tests, through greenhouse studies and finally to field conditions is a challenging task in the selection or elaboration of effective mixtures and inoculation methods to apply beneficial microbes [29]. Studies that contemplate more than one experimental scale are still scarce and it is crucial to understand the biases of inoculation performance, since the beneficial effects of microbial inoculation obtained under greenhouse conditions are not always achieved in the field [30]. To our knowledge, no field studies focusing on seed coating inoculation of chickpea with AM fungi have been reported so far.

In our study it is expected that inoculation of multiple AM fungal isolates results in superior chickpea performance when compared to single AM fungal isolate under both greenhouse and field conditions. Therefore, the objectives of this work were (1) to compare the efficiency of inoculation of single and multiple AM fungal isolates via seed coating and their effects on chickpea yield and nutritional content under greenhouse and field conditions, and (2) to verify whether results from AM fungal inoculation obtained under greenhouse conditions can serve as an indicator of their potential benefits for field applications.

2. Materials and Methods

2.1. Arbuscular Mycorrhizal Fungal Inocula and Seed Coating

Two different AM fungal inocula (provided by Symbiom Ltd., Lanškroun, Czech Republic) were used, one consisted of a single fungal isolate *Rhizophagus irregularis* BEG140 and the other was a mixture of equal proportions of five *R. irregularis* isolates namely BEG141, BEG236, DAOM 197198, KW and AS. Both fungal inocula were grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as the host plant.

For the seed coating treatment, seeds were dusted with *R. irregularis* inoculum (sieved through 500 µm mesh) followed by biochar (0.25% of seed weight) (Ecochar, Ibero Massa Florestal, UI, Portugal). Gum arabic solution (2%) was used as a binding agent. Chickpea seeds were dressed using a rotating pan according to Scott et al. [31]. Twenty AM fungal propagules were applied per seed and estimated by the most probable number [32].

2.2. Experimental Design

The experimental design involved three treatments, resulting from three different inoculations including (1) non-coated and non-inoculated controls (control), (2) plants coated with *R. irregularis* BEG140 (Rcoat), and (3) a mixture of *R. irregularis* isolates (MRcoat). The effect of seed coating with different fungal combinations was evaluated under both greenhouse and field conditions. Both experiments were conducted simultaneously from April to August 2018. The seeds of chickpea (*Cicer arietinum* L. cv. Elixir) were obtained from the collection of the National Institute for Agrarian and Veterinary Research (INIAV).

The field experiment was conducted at the INIAV station in Elvas, Portugal (38°55′07.8″ N, 7°05′33.2″ W, 209 m above sea level). The field had been used for chickpea and oat production in a crop rotation system. The temperature fluctuated from 6 to 40 °C (average 13 to 26 °C), 42 to 78% of relative humidity (RH) and 0 to 8 mm of precipitation. The soil had a clay texture with pH (1:2.5 *w/v* water) 7.5, electrical conductivity 0.30 mS.cm⁻¹, 2.1% organic matter, 168 mg kg⁻¹ extractable (Egner-Riehm) phosphorus (P), >200 mg kg⁻¹ extractable (Egner-Riehm) potassium (K), 7174 mg kg⁻¹ extractable (ammonium acetate) calcium and 206 mg kg⁻¹ extractable (ammonium acetate) magnesium. Eight months before starting the field experiment, the soil was amended with 200 kg ha⁻¹ of fertilizer with 20% N; 8% P and 10% K (NERGETIC C-PRO 20-8-10[®], ADP Fertilizantes, Alverca do Ribatejo, Portugal). Each experimental plot consisted of three rows of 4 m (with 30 seeds each and 60 cm between rows) that was organized in a split-plot randomized block with three repetitions per treatment. After the seed coating treatment, seeds were sown manually at 2 cm depth and separated by at least by 13 cm. During the experiment, plants were grown under natural rainfall conditions without receiving further irrigation or fertilization.

For the greenhouse experiment, soil was collected from the same field, sieved (2 mm) and used in order to provide a similar soil microbiota and chemical properties. Ten replicates per treatment were disposed in individual plastic pots of 3 L (14 × 14 × 23 cm) that received one seed and were arranged in a fully randomized scheme. The pot positions were periodically swapped in order to minimize specific differences related to microsite location in the greenhouse. During the experiment, greenhouse temperature ranged from 14 to 42 °C (average 16 to 30 °C) and RH was maintained between 40 to 85%, with an average photoperiod of 12 h. In order to maintain soil humidity, pots were irrigated as frequently as required to restore water losses produced by evapotranspiration, on average 3 times a week.

2.3. Plant Measurements

In both field and greenhouse experiments, plants were harvested approximately 120 days after sowing (DAS). DAS required for germination, flowering (flowering of 50% of the plants) and maturity (maturity of 50% of the plants) were recorded. Pods and seeds were collected, counted and weighted to quantify grain yield per plant. Shoot samples from both experiments were dried at 70 °C for 48 h and weighed. For plants grown under field conditions, the weight of 100 seeds, harvest index of chickpea [Grain dry weight (GDW)/Shoot dry weight (SDW)] and the relative effectiveness (RE) of inoculation (SDW of inoculated plants/SDW non inoculated plants) were also calculated according to Maâtallah et al. [33].

2.4. Crude Protein and Fiber, Fat and Ash Grain Content Analyses

After collection, grain samples were dried at 70 °C for 48 h and finely ground. The protein content was analyzed according to the Kjeldahl method (ISO 20483:2006). Crude protein was calculated by multiplying the N content by 6.25. The crude fiber content was quantified using the method with intermediate filtration from the Portuguese Norm (NP) EN ISO 6865:2009. After acid and alkaline digestion of the sample, the crude fiber content was calculated from the loss in mass resulting from ashing of the dried residue divided by the mass of the test portion. Finally, the grain fat content was determined with ether ethylic using the extraction apparatus Soxtec System HT1043 in accordance to the NP 876:2001. The ash yield was determined by incineration and calculated as a fraction of the mass of ashing dish and incinerated residue, divided by the mass of the test portion, according to the international standard ISO 2171:2007.

2.5. Mycorrhizal Development

After harvest, roots from plants collected from greenhouse and field experiments were separated from shoots, gently washed tap water, cut into 1-cm pieces and stained with trypan blue using a modified

Phillips & Hayman [34] protocol [35]. The percentage of root length colonized (RLC) was assessed by the grid-line intersect method [36] under a stereomicroscope (Leica EZ4 HD, Wetzlar, Germany).

2.6. Statistical Analysis

Normality and homogeneity of variances were confirmed and data analyzed using one-way analysis of variance (ANOVA). In the case of pod number and the number of pods with 2 grains, square (x^2) and root (\sqrt{x}) transformations were required to satisfy normality assumptions before ANOVA. When a significant F -value was obtained ($p < 0.05$), treatment means were compared using Duncan's multiple range test. When normality assumptions were not met (as in the case of SDW, the weight of individual grains and ash content for field data and grain number and protein content for greenhouse data), differences between groups were compared using non-parametric Kruskal–Wallis test. SPSS 25.0.0 software package (IBM SPSS Statistics, Armonk, NY, USA) was used to perform all the statistical analyses.

3. Results

3.1. Growth Parameters

In general, chickpea seeds took approximately double time to germinate in the field (15 DAS) compared to the germination observed in the greenhouse (7 DAS). Nevertheless, seed coating with AM fungi did not affect germination rates of chickpea. Flowering and maturation times of cowpea plants were similar under greenhouse and field conditions; in the greenhouse, flowering and maturation took 46 and 101 DAS, whereas 42 and 102 DAS were necessary under field conditions, with no significant differences between inoculation treatments.

Under greenhouse conditions, single inoculation of *R. irregularis* BEG140 (Rcoat) did not show clear effects on chickpea plants when compared to control, with the exception of the grain individual weight. However, seed coating with the mixture of *R. irregularis* isolates (MRcoat) showed positive effects on chickpea productivity when compared with control treatment at both experimental scales (Table 1). Under greenhouse conditions, plants treated with the MRcoat treatment exhibited a significant increase in SDW (14%, $p < 0.001$) and also in the grain individual weight per plant (21%, $p < 0.05$) for. In the field, the effect of the coating treatment containing the *R. irregularis* consortia was much more noticeable. Here, MRcoat treatment produced a significant increase in valuable agronomic parameters as the number of pods per plant, seeds per pod and seeds per plant in comparison with the remaining treatments. Inoculation significantly enhanced the number of pods and grains by 160% ($p < 0.001$) and 148% ($p < 0.001$), respectively.

Table 1. Growth and productivity parameters of chickpea in different inoculation treatments (control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat)) under greenhouse and field conditions.

Experimental Scale	Treatment	SDW (g)	Number of Pods Per Plant	Number of Pods with 2 Grains Per Plant	Number of Grains Per Plant	Weight of Individual Grains Per Plant (g)	Grain Yield Per Plant (g)
Greenhouse	Control	1.4 ± 0.1 a	8 ± 0.6		5 ± 0.2	0.29 ± 0.0 a	1.5 ± 0.0
	Rcoat	1.3 ± 0.0 a	7 ± 0.1	NA	5 ± 0.4	0.33 ± 0.0 b	1.5 ± 0.1
	MRcoat	1.6 ± 0.1 b	8 ± 0.9		4 ± 0.3	0.35 ± 0.0 b	1.5 ± 0.1
Field	Control	10.2 ± 2.1	24 ± 4.1 x	4 ± 0.6 x	27 ± 3.8 x	0.30 ± 0.0	8.4 ± 1.4 x
	Rcoat	6.0 ± 1.1	25 ± 7.6 x	3 ± 1.4 x	25 ± 7.9 x	0.33 ± 0.0	7.9 ± 2.5 x
	MRcoat	8.9 ± 0.9	62 ± 1.6 y	9 ± 1.9 y	68 ± 5.9 y	0.30 ± 0.0	20.1 ± 1.4 y

Means (± 1 SE) followed by letters that indicate significant differences between treatments within the same experimental scales according to Duncan's multiple range and Kruskal–Wallis test at $p < 0.05$. SDW, shoot dry weight. NA, not applied.

The grain yield of chickpea was not affected by AM fungal inoculation when grown under greenhouse (Table 1). On the other hand, MRcoat treatment significantly increased grain yield per plant (140%, $p < 0.001$) under field conditions. Consequently, harvest index was also significantly higher in the MRcoat treatment (Table 2). There were no significant differences between treatments in the weight of 100 seeds (32 g for all treatments).

Table 2. Harvest index (ratio of grain dry weight to shoot dry weight) of chickpea and relative effectiveness of inoculation (ratio of shoot dry weight of inoculated plant to shoot dry weight of non-inoculated plants) under field conditions.

Treatment	Harvest Index (%)	Relative Effectiveness (%)
Control	103.7 x	-
Rcoat	147.4 xy	66.0
MRcoat	229.8 y	107.0

Means followed by letters that indicate significant differences between treatments according to Duncan's multiple range test at $p < 0.05$. Rcoat (*Rhizophagus irregularis*), MRcoat (mixture of *R. irregularis* isolates).

A summary of the chickpea productivity parameters among the different treatments under greenhouse and field conditions is presented in Figure 1.

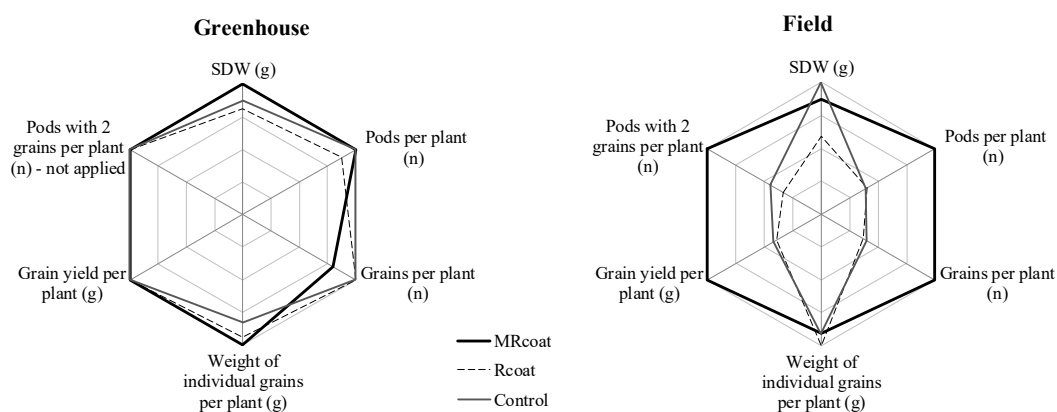


Figure 1. Representation of productivity parameters of chickpea in different inoculation treatments (control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat)), under greenhouse and field conditions. Radial graphs represent results relative to the higher value (indicated as 100%) for each productivity parameter.

3.2. Grain Quality

In terms of nutritional quality, no significant differences in crude protein, fat, crude fiber and ash content of chickpea grains were detected in the greenhouse trial (Table 3). Under field conditions, no significant differences in protein, fat and ash grain content were detected among treatments with the exception of crude fiber that was significantly higher in non-inoculated plants.

3.3. Mycorrhizal Colonization

The percentage RLC of plants grown in the greenhouse was not statistically different among treatments, with values of 65%, 66% and 74% for control, Rcoat and MRcoat, respectively (Figure 2). Under field conditions, MRcoat treatment showed higher rates of fungal colonization in their roots, 69% of RLC in comparison with 54% and 42% in control and Rcoat treatments, respectively.

Table 3. Crude protein, fat, crude fiber and ash content of chickpea grains in different inoculation treatments (control, *Rhizophagus irregularis* (Rcoat), mixture of *R. irregularis* isolates (MRcoat)) under greenhouse and field conditions.

Experimental Scale	Treatment	Crude Protein (%)	Fat (%)	Crude Fiber (%)	Ash (%)
Greenhouse	Control	19.9 ± 0.4	4.2 ± 0.1	4.5 ± 0.4	2.5 ± 0.1
	Rcoat	19.3 ± 0.3	4.6 ± 0.3	4.4 ± 0.2	2.4 ± 0.1
	MRcoat	18.4 ± 0.5	4.5 ± 0.2	4.6 ± 0.6	2.3 ± 0.1
Field	Control	20.8 ± 0.5	3.9 ± 0.1	4.1 ± 0.2 y	3.0 ± 0.2
	Rcoat	20.8 ± 0.2	3.9 ± 0.2	3.5 ± 0.1 x	2.9 ± 0.0
	MRcoat	20.6 ± 0.5	3.9 ± 0.3	3.1 ± 0.1 x	2.6 ± 0.0

Means (±1 SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test and Kruskal-Wallis test at $p < 0.05$.

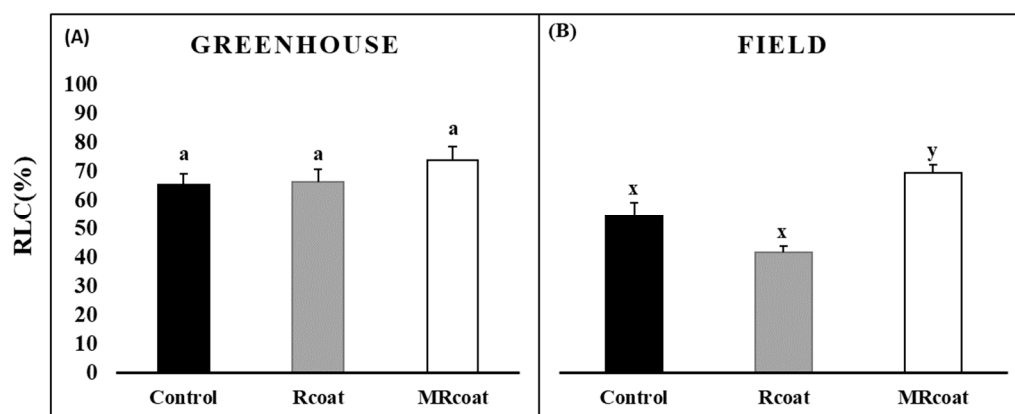


Figure 2. Percentage of root length colonization (% RLC) of chickpea non-inoculated (control) and inoculated with *Rhizophagus irregularis* BEG140 (Rcoat) or mixture of *R. irregularis* isolates (MRcoat) via seed coating in greenhouse (A) and field (B) trials. Columns are means ± 1 SE and letters indicate significant according to Duncan's Multiple Range test at $p < 0.05$.

4. Discussion

Difficulties to replicate in the field the effects observed under controlled greenhouse conditions are generally considered a major constrain to expand the use of beneficial microbes in agriculture. In order to address this pitfall, experimental conditions such as time of the year, soil, microbial inoculum and concentration, plant cultivar, seed origin and inoculation method were kept identical in both greenhouse and field experiments. Our results showed that the use of *R. irregularis* mixture (MRcoat) as a seed coating treatment has great potential to promote chickpea production, with significant increases in grain yield of plants grown in the field. Previous studies had already indicated that the inoculation of AM fungi can be used to improve biomass and productivity of chickpea [6,37,38], but with lower grain yield and harvest index, when compared to those obtained in plants inoculated with multiple *R. irregularis* isolates in our field trial. Harvest index is frequently used as an indicator of yield efficiency and consequently as a selection criterion for crop breeding [39]. In our study, plants inoculated with the mixture of AM fungi were very effective and capable of producing a higher amount of grains with less shoot biomass. Contrary to previous reports [38,40], AM fungal inoculation did not influence the weight of 100 seeds.

Taking into account the grain yields obtained in our study (see Table 1), the producer price of chickpea in Portugal (1.10 USD/Kg, according to FAOSTAT [41]) and the cost of seed coating (132.34 USD/ha, including materials and labor) we estimated the profit obtained for the different treatments. Since there was no cost of seed coating in the control treatment, the obtained profit was 385.00 USD/ha. For the Rcoat and MRcoat treatments the estimated profits were 229.74 and

788.91 USD/ha, respectively. This shows that, despite the inoculation costs, choosing the right inoculum for seed coating can result in a substantial gain for the farmer.

Despite the common presence of AM fungi in agricultural soils, seed inoculation with selected isolates can increase plant root colonization and crop productivity [6,42,43]. All field-grown plants, including non-inoculated controls, presented mycorrhizal root colonization, due to the ability of native fungi to colonize plant hosts. However, when compared to non-inoculated controls or plants inoculated with single fungal isolate, inoculation with multiple AM fungi increased root colonization and plant productivity. It is well known that the interactions among different AM fungal isolates can be synergistic, neutral or antagonistic [44–46]. Thus, inoculation of plants with non-native AM fungal isolates does not necessarily produce beneficial effects, as competition with native AM fungal species or even between selected species can occur. Soil physicochemical properties can also have a strong impact on the symbiotic relationship between plants and fungi [18]. *R. irregularis* BEG140 was selected for this study due to the promising effects in increasing chickpea biomass and grain yield obtained in previous greenhouse trials [7]. Yet, the soil used in the above-mentioned experiment was sterilized (free of native AM fungi and remaining soil biota) and had different physicochemical characteristics from the soil used in this study, which had relatively high available P content. Our findings suggest that competition with native AM fungi or soil physicochemical status might have influenced the symbiotic relationship between *R. irregularis* BEG140 and chickpea, contrary to the MRcoat treatment where the combined use of *R. irregularis* isolates produced larger beneficial effects on plant growth. In general, it is considered that the combined use of soil microbes with different attributes provide extra benefit due to the combination and complementarity of different mechanisms of action [21]. Among other factors, the observed positive effects could be also due to the expansion of environmental niche for mycorrhiza functioning [44,47]. Further investigation would be needed to evaluate which AM fungi isolates in the treatment MRcoat were active and responsible for the benefits.

Besides increasing plant productivity, inoculation of AM fungi can improve plant and/or seed nutrient content [5–7]. However, our results showed no enhanced nutrient content of grains produced by inoculated plants. The exception was the higher grain crude fiber content in non-inoculated chickpea grown under field conditions, a fact that has been previously reported [48,49].

Despite the frequent demonstration of efficacy in laboratory and greenhouse experiments, the inconsistency of effectiveness or the lack of field data regarding AM fungi inoculation is still one of the main restraints for its wide application [22,50,51]. According to the meta-analysis of Zhang et al. [52] there is a bias favoring controlled conditions for AM fungi inoculation; laboratory studies including inoculated crops tend to lead to higher grain yield increase in comparison with those studies carried out in the field. Surprisingly, our work contrasts these data as it shows that the positive effect of multiple AM fungi inoculation is maximized under field conditions.

Although direct comparison between results obtained under greenhouse and field conditions would be troublesome due to limiting aspects and interacting factors of the experimental procedure (e.g., constraining of roots within pots, root density/root system architecture and water requirements) [16,53], both greenhouse and field scale are necessary and can be used as an indicator of potential positive effects. For instance, in field trials, Colla et al. [54] and Roupael et al. [24] successfully based their seed coating formulations on results obtained under greenhouse conditions where the AM fungal isolates had a positive influence on the growth, yield and nutrition of different plants species (zucchini, lettuce and winter wheat). Our results showed that the same treatment (MRcoat) was able to benefit chickpea performance both under greenhouse (plant shoot dry weight and seed weight increase) but especially under field conditions (pod and grain yield improvement). The above mentioned limiting aspects such as pot and root size/depth or environmental factors (e.g., water, temperature) under greenhouse conditions, might have exerted a different influence on the AM fungi colonizing chickpea roots than in the field. This could have led to the observed differences in %RLC between treatments obtained under greenhouse and field conditions (Figure 2).

5. Conclusions

The selection of AM fungal isolates that relates to host plant and crop growing conditions is essential to achieve good mycorrhizal efficiency and to obtain economical profits from crop productivity. Summarizing the main results obtained in this study, the application of multiple AM fungal isolates seemed to be a potential strategy to boost chickpea productivity, when compared to the inoculation of single AM isolate. Seed coating can be an appropriate tool to deliver AM fungi and the combined use of multiple isolates exhibited benefits for chickpea plants at different experimental scales, but the effect was especially relevant under field conditions. To our knowledge, this is the first field evidence of improved enhanced yield of chickpea inoculated with AM fungi via seed coating. Although greenhouse trials represent a prospective indication of microbial field-application potential, results are not necessarily representative in each case. In this sense, information provided by the combination of greenhouse and field trials is highly valuable and the simultaneous approach should be considered for further experimental designs. Integrating AM fungi into agricultural systems via seed coating in order to increase grain yield of crops is a potential valid approach for sustainable agriculture.

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Conflicts of Interest: The authors declare no conflict of interest.

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