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Arbuscular Mycorrhizal Fungus *Funneliformis mosseae* Improves Soybean Growth Even in Soils with Good Nutrition

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Abstract: Arbuscular mycorrhizal fungi (AMF) improve plant growth and may be useful in maintaining and even restoring soil. However, data on the latter function are sparse and only indirect, which is especially true for conventional management conditions with adequate nutrient availability. Our study focused on utilizing the prevalent AMF species, *Funneliformis mosseae*, to enhance *Glycine max* production, while also exploring its partly explored impact on soil aggregation. Working in greenhouse conditions, we examined whether, in a nutrient-sufficient environment, AMF would improve crop biomass accumulation and nutrition, as well as the stability of soil aggregates (SAS). We also looked for a synergistic effect of dual inoculation using AMF and symbiotic rhizobium. Plants were or were not inoculated with AMF or *Bradyrhizobium japonicum* in a two-factorial design. AMF inoculation increased soybean biomass, but AMF inoculation had no impact on P and N input to the shoots. Mycorrhiza did not affect either glomalin abundance or SAS. All the impacts were, however, independent from rhizobial inoculation, which was ineffective in this nutrient-available environment. Our assay suggests that arbuscular mycorrhiza may have a positive effect on soybean growth even under conventional management with adequate nutrition. The positive effects of AMF on soybean growth, together with the fact that AMF generally do not thrive in good nutrient availability, should be taken into account when planning mineral fertilization levels.

Keywords: agricultural soils; *Funneliformis mosseae*; plant nutrition; soybean *Glycine max*; stability of soil aggregates; tripartite symbiosis

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1. Introduction

Arbuscular mycorrhizal fungi (AMF; Glomeromycotina [1]) are key microorganisms in soil [2]. They live in symbiosis with a great majority (>90%) of plant species, including many important crops [3,4]. Being soil as well as plant symbionts, they create interfaces between plants and the soil environment. AMF bring many benefits to the host plant and perform many critical functions necessary for the functioning of ecosystems. They contribute significantly to plant nutrition (especially of P and N) and increase the productivity of agricultural crops [5–8]. They benefit plants by mitigating abiotic stress [9–11] and protecting against pathogens [12–16]. The extraradical mycelium of AMF is primarily responsible for a major part of the P acquired by the plant [17]. AMF may be responsible for as much as 100% of the P in plant biomass [18,19]. Plants seem to rely on AMF for P supply once colonized, as the formation of mycorrhizae can lead to the downregulation of plant P transporters [18–20]. In return, as much as 20% of host photosynthates can be allocated to the fungus [21].

AMF also improve plant yields indirectly through their effects on soil aggregation [22–25], which is a critical issue for sustainable agricultural development and ecosystem functioning. AMF are important producers of extracellular polymeric substances—glomalin—that

contribute to the aggregation of soil particles [26,27], maintain ambient moisture levels, and serve as a reserve of carbon and mineral nutrients [28]. The extraradical mycelium of AMF then links colonized roots with the soil matrix [29], entangles soil particles, and holds them together [30]. Furthermore, the microsymbionts change the chemical composition of root exudates [12]. These changes, together with the extraradical mycelium of AMF, which physically modifies the soil environment and serves as a carbon source to other soil microorganisms, lead to compositional changes in soil microbial communities [12–31] that may indirectly affect aggregation. What benefits are realized, however, and to what extent AMF symbiosis brings those advantages to the host or the ecosystem depends upon environmental conditions, as well as abiotic and biotic interactions.

In natural environments, most legume roots form so-called tripartite symbiosis [32,33], meaning that they are colonized simultaneously by AMF and by symbiotic rhizobia that interact not only with the host plant but also with each other [33]. Rhizobial nodules require P for reducing the N_2 molecule to two NH_3 molecules [34], whereby they supply N to their host plant. Because AMF are able to improve the P nutrition of legume plants, AMF can increase N fixation rates by root nodules [33]. The plant, in turn, can allocate C from photosynthesis between symbiotic partners according to what and how many benefits they bring, thereby affecting their abundance [35,36]. Additionally, AMF increase the abundance of rhizobiales in the soil [37]. These are thus complex relationships, and it is not clear what will result from the interaction among the host legume, rhizobium, and AMF. Nevertheless, it is generally believed that benefits multiply when these organisms work together [12] in their tripartite symbiosis. AMF and rhizobia, for example, have been shown to interact to improve plant productivity, seed yield, P and N acquisition, and photosynthetic rates [31,38–41].

A question remains as to just what is the function of AMF in agricultural ecosystems under conventional management. We can expect nutritional benefits especially in soils whose productivity is limited by a lack of P [42]. However, intensive agriculture is based upon supplying the main nutrients in such quantities that they are not limiting for plant growth. It therefore offers only a small role for the uptake of nutrients through symbiotic fungi. Other, non-nutritional benefits of AMF symbiosis for the plant and ecosystem can nonetheless still manifest here, such as heightened resistance to stress or increased stability of soil aggregates. It is generally assumed, however, that in soils with a sufficient content of nutrients, especially P, the plant can reduce the root colonization by AMF [8,43] because they bring no significant nutritional benefits and become energy demanding upon the host plant [43]. The level of the plant's dependence upon mycorrhizal symbiosis as well as the already-mentioned non-nutritional benefits can play roles here. AMF also can survive in plant roots as commensals, and their extraradical mycelium can still be beneficial to soil quality, albeit to a lesser extent.

Soybean (*Glycine max* (L.) Merr.) is one of the most widely cultivated crops throughout the world under various climates [44]. It forms associations with AMF and rhizobia simultaneously [45,46]. Among legumes, soybean ranks first in the production of proteins and fats per unit growing area. Soybean farmers are increasingly adopting alternative management strategies to improve the sustainability and profitability of growing this crop. Microbial symbionts may be useful here [47,48], but their potential use must be preceded by research as to their effects in different conditions of agricultural ecosystems, including the context of interactions between microorganisms.

The objectives of the present study were to ascertain the effects of AMF, independently and in dual inoculation with rhizobium, in the conditions of conventionally managed agricultural soils with good nutrient availability, on (i) the growth and nutrition of *soybean*, and (ii) soil quality as expressed by water stability of soil aggregates (SAS). We hypothesized that, in soils with good nutrition, the net effect of AMF on plant biomass accumulation and nutrition may be zero, even though they occur and proliferate in plant roots. At the same time, however, the AMF may receive sufficient organic nutrition from their hosts to support the production of glomalin by their extraradical mycelium, thus increasing SAS. In

the presence of rhizobium, AMF compete in a nutrient-rich environment for the C from the host plant; as a result of this, their abundance decreases, thus decreasing their contribution to SAS.

2. Materials and Methods

2.1. Experimental Design

The experiment was a fully factorial design with two factors: (1) AMF inoculation (inoculated with AMF [M+] or not so inoculated [M−]), and (2) rhizobial treatment (inoculated with rhizobium [R+] or not so inoculated [R−]). In this way, 4 treatments were obtained: M−R−, M−R+, M+R−, and M+R+. Five replicate pots were established per treatment combination.

2.2. Cultivation Pots and Substrate

Pots 1.5 L in volume were lined with a plastic mesh (1.2 mm opening) at the bottom, sterilized with 96% ethanol, and then filled with soil. The soil was thoroughly mixed, γ-irradiated (>25 kGy) field soil (Orthic Luvisol, pH 6.82, 7.7×10^{-3} g/kg water-extractable P, 101.6×10^{-3} g/kg total P, 8.3×10^{-3} g NH_4/kg, 9.3×10^{-3} g NO_3/kg, 1.1 g total N/kg, 11.2 g total C/kg C, 0.28 g oxidizable C/kg, 99.1×10^{-3} g Mg/kg, 126.3×10^{-3} g K/kg, and 2148×10^{-3} g Ca/kg, 20.9% SAS) from Hněvčeves, Czech Republic (15°43′3″ E, 50°18′47″ N). The soil was homogenized by sieving through a mesh with a 10 mm opening size.

2.3. Microbial Inoculation

Half of the pots (M+ pots) were supplemented with 25 g of mycorrhizal inoculum. The inoculum consisted of potting substrate from previous pot cultures of *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & Schuessler (2010) BEG 161, a generalist symbiont, widespread all over the world in almost all soils and climatic zones [4]. BEG is an abbreviation for the International Bank for the Glomeromycota (www.i-beg.eu, Accessed on 10 February 2020). The other half of the pots (M– pots) each received 25 g of the same but sterilized substrate and 10 mL filtrate of complex mycorrhizal inoculum. The filtrates were prepared through vigorous agitation (30 min) of the respective substrate suspension in sterile distilled water (7:10, *w*/*w*) and filtration through two layers of filter paper to provide for similar microbial communities potentially confounding non-mycorrhizal biotic effects as M+ pots. The solid M+ and M− inocula were added 2 cm beneath the surface of the potting substrate.

Rhizobial strain *Bradyrhizobium japonicum* (Kirchner 1896) Jordan 1982 R697 came from the Collection of Soil Bacteria of the Culture Collection of Microorganisms (VURV-R) at the Crop Research Institute, Prague, and was applied to each pot with 15 mL of sterile water (7.2×10^{-9} CFU per 1 mL) in immediate vicinity of the seeds.

2.4. Model Plant, Plant Cultivation, and Harvest

The model plant was *Glycine max* (L.) Merrill. var. Edamame. The seeds were surface-sterilized (75% ethanol) and thereafter rinsed with sterilized tap water. Four seeds per pot were directly sown 2 cm beneath the surface of the potting substrate. After 2 weeks, the plants were thinned to 2 seedlings per pot. Plants were grown during spring (April–June) 2020 in a greenhouse at the Crop Research Institute, Prague with average day and night temperatures 26 °C and 21 °C, respectively. The temperatures were automatically regulated by central heating and active fan-facilitated ventilation. The day length was extended to 12 h, with supplemental lighting (metal halide lamps, 250 W each) providing a minimum photosynthesis flux density of 200 μmol m^{-2} s^{-1}. Plants were watered daily. Our study, however, complies with relevant institutional, national, and international guidelines and legislation on handling plant material.

The replicate pots of all the four treatments were randomly placed on a 2 × 2 m surface of greenhouse table and their positions were weekly changed in a random manner to avoid systematic effects of possible uncontrolled environmental factors.

After 57 days, when all the plants had begun to bloom, the shoots of all target plants were cut at the hypocotyl–root interface and subsequently dried for 4 days at 65 °C to determine shoot dry weight, as well as shoot N and P concentrations. All assessments were calculated for the entire pot (2 plants). The roots were washed from the substrate under cold tap water and nodule numbers were counted for each root under a magnifying glass. Further, the roots were cut into 1.5 cm fragments and kept in 50% ethanol until AMF colonization was determined. Soil samples were air-dried and sieved through meshes of different opening sizes. A fraction with soil grain size 1–2 mm was used for assessing water stability of soil aggregates.

2.5. Analyses and Calculations

To evaluate the benefits provided by the microsymbionts to the host plant, shoot dry biomass as well as P and N concentrations were assessed. N concentrations in shoot dry biomass were determined after combustion of the organic matter with subsequent analysis of the generated gases using a Vario elemental CNS analyzer (Elementar Analysensysteme, Langenselbold, Germany). P concentrations were determined in the mineralization solution of dry shoot biomass after decomposition in nitric acid with the addition of hydrogen peroxide in a closed high-pressure microwave system via inductively coupled plasma atomic emission spectroscopy using an Integra XL device (GBC Scientific Equipment, Dandenong, Australia).

Soil used in the experiment was also characterized: soil was first air-dried at room temperature, sieved through 2 mm mesh, then homogenized. Soil pH was assessed in a water slurry (1:5, *w*:*v*) following shaking of the samples for 1 h. Available P, K, Mg, and Ca were assessed according to the Mehlich III method [49] on an Agilent ICP-OES 5110 VDV instrument. NO_3 and NH_4 were determined using calcium chloride solution as extractant according to ISO 14255:1998 on a SKALAR automated chemistry analyzer (Breda, The Netherlands). Soil oxidizable carbon was determined via sulfochromic oxidation according to ISO 14235:1998. Total organic C and N were assessed using the Vario elemental CNS analyzer.

The wet-sieving method of [50] was used to assess SAS (1–2 mm) using HERZOG laboratory equipment (Adolf Herzog, Vienna, Austria) with sieving time of 5 min and 3 repetitions per sample.

To disqualify AMF colonization in M− treatments and to assess AMF abundance in roots of host plants, the extent of root length colonized by AMF hyphae was assessed microscopically using the magnified intersection method in accordance with [51] after staining the roots with trypan blue following [52]. To quantify AMF in M+ roots and soil samples, we evaluated the copy number rRNA gene as a proxy of AMF fungal biomass quantity [53,54]. For this, we extracted DNA from soil samples dried from ethanol solution using a DNeasy Power Soil DNA isolation kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and from dried roots using a NucleoSpin Plant II kit (Macherey-Nagel, Allentown, PA, USA). The evaluation of the rRNA gene copy numbers was then carried out via qPCR reactions with Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA, USA). We used the primers NS31 [55] and AML2 [56] and the thermal program as follows: initial denaturation at 95 °C (3 min) and 55 cycles of denaturation at 95 °C (10 s), annealing at 60 °C (15 s), and elongation at 72 °C (25 s). The primers were synthesized and high-performance liquid chromatography-purified at Generi Biotech (Hradec Králové, Czech Republic). The calibration curve was created via measurement of standards from a dilution series ranging from 10^8 to 10^4. Standard fragment length was 550 bp and concentration of the standard was 77 ng/µL. All samples were related to the weight of 1 g. The analyses were performed on a Roche LightCycler 480 System.

Abundance of total glomalin was assessed in soil samples according to [57] by autoclaving the soil in alkaline citrate solution to yield total fractions and then quantifying it using the nonspecific colorimetric assay according to [58].

2.6. Statistical Analyses

Analyses of variance (ANOVA) with $p < 0.05$ as the significant differences level were calculated in the R 4.2.0 statistical environment [59] after checking for data conformity with ANOVA assumptions (i.e., normality and homogeneity of variances).

Dry biomass, AMF colonization (assessed both microscopically and using qPCR), and number of nodules were log-transformed before the analyses.

To assess effects of the symbioses on the host plant and soil characteristics, we conducted two-way ANOVAs with factors including arbuscular mycorrhizal and rhizobial inoculations against dry biomass, P and N concentrations in shoot biomass, total glomalin, and SAS.

To assess the effects of rhizobial and/or AMF inoculations on the AMF and/or rhizobial abundance, respectively, we performed one-way ANOVAs with the respective inoculation as a factor on AMF colonization of roots and abundance of AMF gene copies in roots and soil, as well as on the nodule numbers.

Mean values and standard errors per treatment combination are presented in the text and figures. As a significant interaction was never revealed between the two factors studied, the mean values calculated over both levels of the other factor are always presented in the case of 2-way ANOVA.

3. Results

3.1. Plant Biomass and Mineral Nutrition

Plant biomass was significantly increased and P and N concentrations were significantly decreased by mycorrhizal inoculation (Table 1, Figure 1). The effect of mycorrhiza was independent from rhizobial inoculation, however, as indicated by insignificant interaction between the effects of the inoculant types on the mentioned plant parameters (Table 1).

Table 1. Significance of effects of arbuscular mycorrhizal fungi (AMF) and/or rhizobial inoculation, and, when observed, their interaction as revealed by one- or two-way ANOVAs on shoot biomass of *Glycine max*, shoot phosphorous (P) and nitrogen (N) concentrations, stability of soil aggregates (SAS), total glomalin, AMF root colonization, number of AMF gene copies in roots of *G. max* and in soil, and number of root nodules. Significant results are shown in bold.

Parameter	AMF Inoculation		Rhizobial Inoculation		AMF × Rhizobial Inoculation	
	F	***p***	***F***	***p***	***F***	***p***
Shoot biomass	14.4	$\mathbf{3 \times 10^{-3}}$	2.9	0.11	0.0	0.96
N concentration	13.2	$\mathbf{0.3 \times 10^{-2}}$	0.6	0.56	0.6	0.59
P concentration	14.9	$\mathbf{0.2 \times 10^{-2}}$	1.6	0.23	0.6	0.44
SAS	0.8	0.38	5.5	**0.03**	3.0	0.10
Total glomalin	1.4	0.26	1.3	0.28	1.6	0.23
AMF colonization	-	-	7.6	**0.03**	-	-
AMF gene copies in roots	-	-	13.2	**0.01**	-	-
AMF gene copies in soil	-	-	0.0	0.94	-	-
Number of nodules	6.2	**0.04**	-	-	-	-

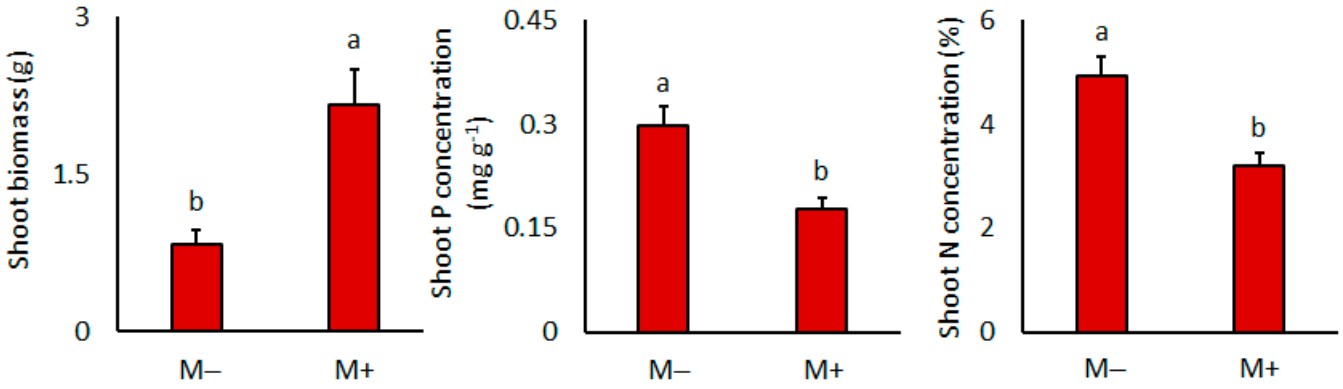

Figure 1. Shoot dry biomass, P and N concentrations in shoots of soybean plant mean per pot as affected by arbuscular mycorrhizal fungi (AMF) inoculation (inoculated with AMF [M+] or not [M−]). As a significant interaction was never revealed between the factors AMF inoculation and rhizobial inoculation when tested with the two-way ANOVAs, the mean values calculated over both levels of the other factor are presented. Bars represent means accompanied by standard errors (n = 10). Different letters above individual bars indicate significant differences between means at $p < 0.05$.

3.2. Stability of Soil Aggregates

There was a significant negative effect of rhizobial inoculation on SAS (Table 1, Figure 2) independent from AMF inoculation (Table 1). Unlike rhizobial inoculation, the AMF inoculation did not affect the SAS significantly (Table 1).

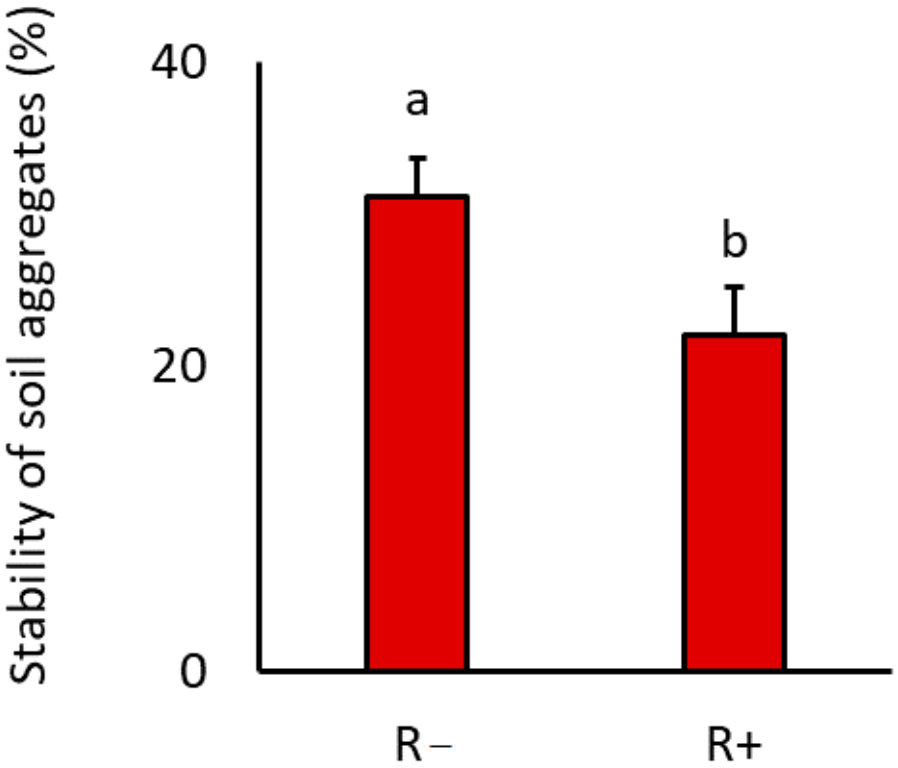

Figure 2. Stability of soil aggregates as affected by rhizobial inoculation (inoculated with rhizobium [R+] or not [R−]). Bars represent means accompanied by standard errors (n = 10). As a significant interaction was not revealed between the factors rhizobial inoculation and AMF inoculation when tested using the two-way ANOVAs, the mean values calculated over both levels of the other factor are presented. Different letters above individual bars indicate significant differences between means at $p < 0.05$.

3.3. Arbuscular Mycorrhizal Fungal and Rhizobial Development

Microscopic observation revealed no mycorrhizal colonization in the roots of M− plants. In the roots of M+ plants (Figure 3), rhizobial inoculation significantly decreased the abundance of AMF as assessed microscopically. This effect was still more pronounced in the case of AMF rDNA gene copy numbers detected using qPCR (Table 1, Figure 3). No effect of rhizobial inoculation was found for the AMF rDNA copy concentrations assessed in soil (Table 1, Figure 3).

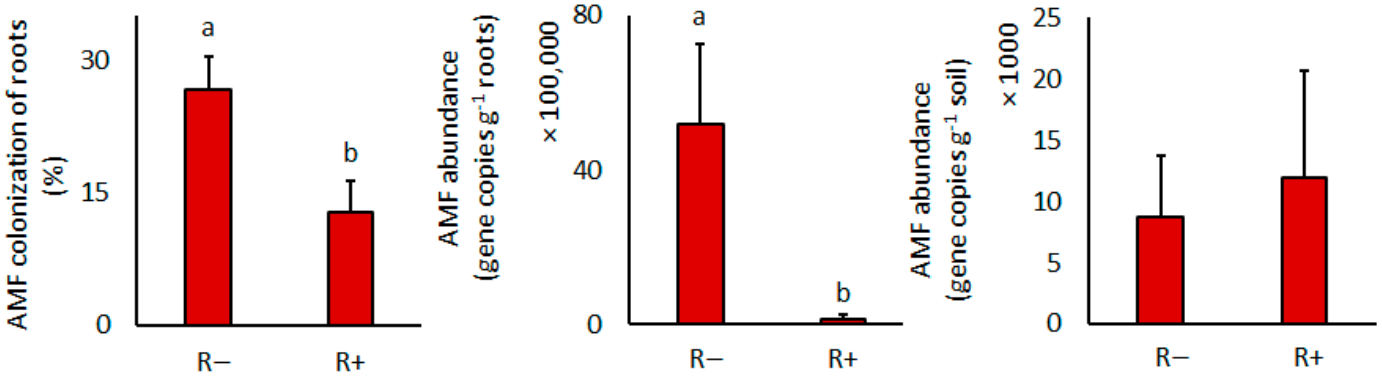

Figure 3. Extent of mycorrhizal colonization of AMF-inoculated roots as assessed under a microscope and using real-time PCR as affected by rhizobial inoculation (inoculated with rhizobium [R+] or not [R−]). Bars represent means accompanied by standard errors (n = 5). Different letters above individual bars indicate significant differences between means at $p < 0.05$.

The roots of R− plants lacked nodules completely. In the roots of R+ plants, the number of nodules was increased by AMF inoculation (Figure 4).

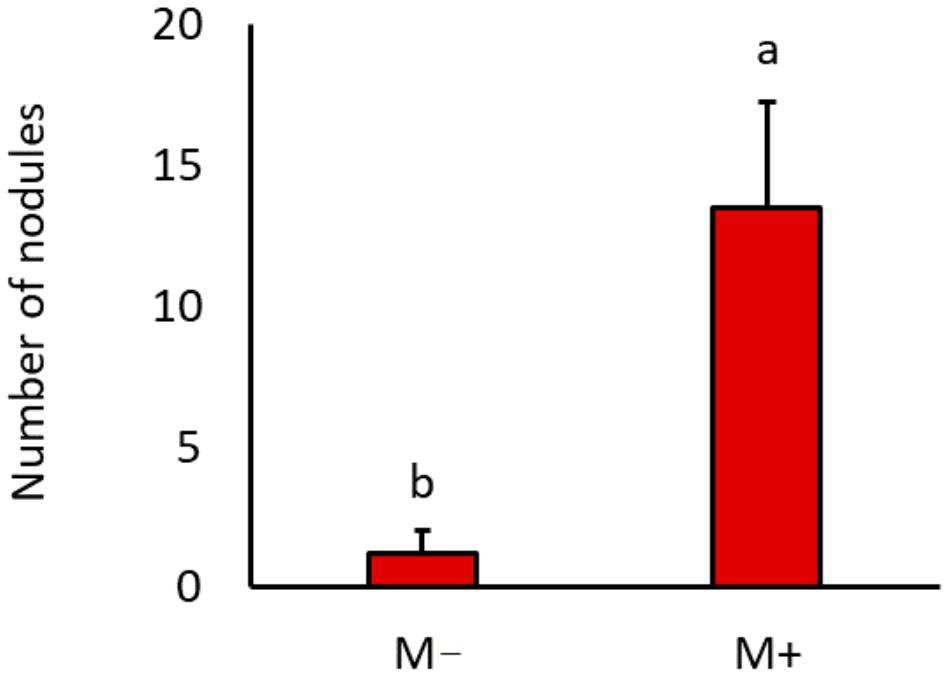

Figure 4. Per pot nodule numbers on roots of soybean as affected by mycorrhizal fungi (AMF) inoculation (inoculated with AMF [M+] or not [M−]). Only roots treated with rhizobium are included. Bars represent means accompanied by standard errors (n = 5). Different letters above individual bars indicate significant differences between means at $p < 0.05$.

3.4. Total Glomalin

The abundance of total glomalin in soil was not significantly influenced by either AMF inoculation or rhizobial inoculation (Table 1).

4. Discussion

Our study shows that mycorrhiza may be established and be beneficial for crop biomass, even in conventionally managed, nutrient-rich agricultural soils. The model crop used here, soybean, increased its biomass as a consequence of AMF inoculation. The increase in soybean biomass was, however, not associated with increased nutrition of the host plants, which is known for soils with low availability of P, or possibly N. This could be a consequence of downregulated root P transporters in the presence of AMF [19,20] and the resulting insufficiency of M+ roots to supply the plant with more P compared with the direct plant P uptake by M− plants in conditions of good P availability. The observed positive effect of AMF on host plant growth in P-rich soils is consistent with the findings of Buil et al. [60], who also found that some of the conventionally managed arable soils allow for the establishment of mutualistic mycorrhiza beneficial for plant growth. Although Buil et al. [60] also showed that AMF in conventional soils may improve plant nutrition, this was true only for some of the soils assessed in the cited study. Similarly, Řezáčová et al. [8] indicated, for one plant species (*Panicum maximum*), increased P uptake by M+ compared with M− plants under conditions of high P fertilization, but they observed no effect for other plant species (*Panicum bisulcatum)*. In our experiment, the increase in plant biomass caused by AMF inoculation was not attributable to improved nutrition, but was probably connected to other, untested effects. Though the effects of AMF on host plant growth are often attributed to enhancement/modification of mineral nutrition or the water supply, important non-nutritional effects have been observed [5] that cannot be interpreted as an alleviation of the nutritional stress applied to plants under experimental conditions. The molecular mechanisms behind these effects are not known but may be due to affection of plant physiology by bioactive molecules produced by the AMF. Nevertheless, the lack of the effect of mycorrhizal inoculation on P and N concentrations in plant shoots may also only be unmanifested due to the lower number of repetitions. Whether it is so or not, our first hypothesis stating that AMF will not be beneficial in highly loaded soils was true here only for the nutritional benefits of mycorrhiza.

In contrast with the literature [61–64], dual inoculation with AMF and rhizobium was not more beneficial for crop biomass than was AMF inoculation alone. In fact, the rhizobial symbiosis was ineffective in this nutrient-rich environment, as evidenced by the low number of nodules and, above all, the lack of N benefits to the host plant.

Yet, there was a negative effect of rhizobial inoculation on AMF abundance and a positive effect of AMF inoculation on nodule numbers (Table 1). These effects can be the result of a negative influence of the host plant on those symbionts that do not bring it sufficient benefits. It is a known fact that if a plant grows in an environment with enough P available and mycorrhiza no longer bring it sufficient P benefits, the mycorrhizal structures in its roots are reduced [33,43,65]. The same is true for N-fixing symbiosis in environments with ample available N [66,67]. If these nutritional benefits are so dispensable for the plant, the C provided to microsymbionts by the plant could become limiting for them [8,68,69] and could lead to competition [33]. This, however, was not reflected in the effect of these microsymbionts on SAS.

The potential of arbuscular mycorrhizal and rhizobial symbioses to influence soil aggregation has previously been described [63,70–72], but here we intended to demonstrate their effect on SAS in a conventional agricultural system with good nutrition. Nevertheless, our second hypothesis considering AMF to be beneficial for soil quality through increasing SAS was not proven. In this regard, we were only able to demonstrate a negative effect of rhizobial inoculation. The absence of an AMF effect on SAS in our experiment is in disagreement with the findings of Heydari et al. [73] and, for example, Zhang et al. [72], who indicated a significant effect of single inoculation with AMF on soil aggregates. This is,

however, consistent with the absence of any AMF effect on the abundance of total glomalin reported from our earlier work as well as with some results from Rillig et al. [74], who indicated carbonates to be the main strengthening agent of the aggregates, not glomalin. Moreover, although the roots of plants were colonized by AMF even in this conventionally managed soil, the levels of root colonization were low (compare Figure 3 with approx. 30–60% and 21–80% root colonization of soybean in [75] and [76], respectively). At the same time, unlike Alami et al. [70] but consistent with Heydari et al. [73], we found no significant increase in SAS caused by single inoculations with rhizobia. As the impact of rhizobial inoculation on SAS was negative and independent from AMF, our experimental evidence did not support the expected functional synergy between the two root symbionts. The decrease in SAS caused by rhizobial inoculation could be connected to its impact on plant exudate production or root morphology [77], but this is beyond the limits of our current research.

Our assay suggests that arbuscular mycorrhiza may improve plant biomass even in conventional agricultural soils with adequate availability of P. Nevertheless, nutrition by AMF was not found to account for this increased biomass production. Notwithstanding, it would be useful to reanalyze and optimize the doses of fertilizers applied in conventional management, because they usually limit the development of AMF mycelia in the roots and in the soil and thus also probably limit the size of the positive effect of mycorrhiza on plant biomass and other ecosystem services such as soil quality. Before that, however, further research on mycorrhizal effects on host plants and SAS in high-loaded systems is needed, taking into account different soil types, so that the results could be generalized.

Author Contributions: V.Ř. planned and designed, and V.Ř., E.N., I.S., A.C. and M.G. carried out the experiment and analyses. V.Ř. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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

Abbreviations

AMF, arbuscular mycorrhizal fungi; M+, inoculated with AMF; M−, not inoculated with AMF; SAS, water stability of soil aggregates.

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