Benefits of dual inoculation with arbuscular mycorrhizal fungi and rhizobia on Phaseolus vulgaris planted in a low-fertility tropical soil

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Short Communication

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Abstract

The growth response of *Phaseolus vulgaris* to dual inoculation with arbuscular mycorrhizal (AM) fungi and rhizobia was studied in a low-fertility tropical soil in Madagascar. Two isolates of AM fungi identified as *Acaulospora* sp. and *Glomus* sp., respectively, along with a cocktail of ten *Rhizobium* spp. strains were used to conduct a greenhouse experiment in a fully randomized block design with two factors. The *Phaseolus vulgaris* seedlings received one of the following inoculation treatments: no inoculation, separate inoculation with each of the three microbial symbionts (the two AM fungal isolates and the rhizobia), and co-inoculation with each of the two AM fungal isolates and the mix of rhizobium strains. The results showed an additive effect of co-infection by AM fungi and rhizobia on plant growth and on the total N content of the plants, along with a synergistic effect on the total P content, the number of nodules and the mycorrhizal rate of the plants. Dual symbiosis with native strains contributes to the success of legumes, especially in harsh environments and low-fertility tropical soils.

Keywords: Arbuscular mycorrhizal fungi; Rhizobia; Dual inoculation; Native strains; low-fertility tropical soil

1. Introduction

The intensive agriculture model, based on the use of synthetic inputs and natural resources to minimize the effects of production-limiting factors and environmental heterogeneity, is gradually giving way to an alternative model based on agro-ecological technologies (Duru and Therond, 2015; Stavi et al., 2016). One such technology relies on biodiversity management within agro-ecosystems to provide supporting and regulating ecosystem services in a manner to improve resource-use efficiency and reduce the negative impacts of conventional agriculture (Duru and Therond, 2015).

Within this biodiversity, soil microorganisms mostly belonging to the bacteria and fungi are involved in complex and diverse forms of interactions with terrestrial plants (van der Heijden et al., 2016). Consequently, plants can no longer be considered as stand-alone biological entities (Vandenkoornhuyse et al., 2015). Their multiple and complex interactions with mutualistic symbionts enable them to derive a wide range of benefits (Ossler et al., 2015; Souza et al., 2015). Indeed, it is widely recognized that soil microorganisms perform crucial roles in nutrient cycling and are involved in key plant functions, such as nutrition and growth (Richardson et al., 2009).

Two of these complex interactions have been widely described: the close association between roots and (i) fungi forming the well-known arbuscular mycorrhizal (AM) fungal association and (ii) bacteria belonging to the genus *Rhizobium* forming the root-nodule with Fabaceae. AM fungi are obligate plant symbionts that provide resources for plants, primarily phosphorus (P), in exchange for plant photosynthates (Smith and Read, 2008). Rhizobia are free-living soil bacteria that colonize the root systems of many legume species and fix atmospheric nitrogen (N) (Peoples et al., 1995). A key meta-analysis found an overall additive effect of co-infection by AM fungi and rhizobia on plant growth responses (Larimer et al., 2010). To our knowledge, few studies have investigated synergistic effects arising from

mixed inoculation of Rhizobium-mycorrhiza (e.g. Young et al., 1988). However, according to Nadeem et al. (2014), synergistic interactions are more likely to be effective in conditions where biotic or abiotic stresses have occurred.

One of the major mechanisms by which inoculation with AM fungus acts on plant functions seems to be phosphatase activity, involving key enzymes responsible for the hydrolysis of organic P (Tabatabai, 1994). Indeed, there is a broad consensus on the limitation of symbiotic N_2 fixation by P availability in terrestrial ecosystems (Augusto et al., 2013) and the lack of adequate levels of available P in tropical soils, such as in Madagascar, is one of the major constraints for crop production (Raminoarison et al., 2020). Despite various studies showing the benefits of the dual inoculation of legumes, such effectiveness in low-fertility tropical soils with a high P sorption capacity is still poorly documented.

The aim of this study was to determine the effects of dual inoculation of both native microsymbionts, rhizobial bacteria and AM fungi, on *Phaseolus vulgaris* growth in a P- and N-depleted tropical soil. Given the complementarity of N and P as plant growth-limiting resources, this study tested the hypothesis that dual inoculation with both symbionts would have a positive interaction in association with plant roots, and would benefit *Phaseolus vulgaris* growth and productivity. Moreover, we assumed that, whereas most of the time AM fungi and rhizobia do not interact synergistically (Larimer et al., 2010), it could be more effective for enhancing N fixation in stressful environments caused by the low availability of P in tropical soils.

2. Materials and methods

We conducted a greenhouse experiment in Madagascar with *Phaseolus vulgaris* L. cv. Ranjonomby as a plant model. A Ferralsol (0-20 cm), typical of the hills of the Malagasy Highlands, was collected at Lazaina (18°46′ S, 47°32′ E, North of Antananarivo) from

93 unfertilized long-term grassland fallows. It was an acidic (pH 5.4) sandy clay loam, with total
94 carbon, N and P contents of 16.4, 0.91 and 0.61 g kg^{-1}, respectively (Henintsoa et al., 2017).
95 Phosphorus availability was very low (Pi_{water} = 1.1 mg P kg^{-1}) due to its sorption by clay
96 minerals and iron/aluminium oxides, which amounted to 244 g kg^{-1} for kaolinite, 247 g kg^{-1}
97 for gibbsite and 36 g kg^{-1} for iron oxides. It was sieved with a 2-mm mesh, mixed (1:1, w/w)
98 with washed river sand (with total C, N and P contents of 0.17, 0.013, 0.002 g kg^{-1}), and
99 autoclaved at 121°C for 40 min.

100 Native strains of rhizobia and AM fungi were isolated from the roots and rhizospheric
101 soil of bean (*Phaseolus vulgaris*), respectively, taken from an experimental design with
102 intercropped bean and rice growing in the same soil at Lazaina. Two AM fungal isolates
103 extracted by the wet sieving and decanting method (Gerdemann and Nicolson, 1963) were
104 selected. They were identified according to their morphological features (colour, size and
105 shape), and by using the key provided by the International Culture Collection of Vesicular
106 Arbuscular Mycorrhizal Fungi (INVAM, http://www.invam.wvu.edu): M1, with 50-µm
107 brown spores, consisting of *Acaulospora* sp. and M2, with 80-µm black spores, consisting of
108 *Glomus* sp. The rhizobial inoculum S1 corresponded to a cocktail of ten *Rhizobium* spp.
109 strains from bioassays carried out on a collection of infective isolates, selected for their
110 greater symbiotic effectiveness.

111 The experiment was a fully randomized block design with two factors, inoculations of
112 AM fungus (coded "M") and rhizobia (coded "S"), and four replications. The design
113 comprised a negative control without inoculation (coded "S0/M0"), and treatments with
114 inoculation by the two AM fungal strains, *Acaulospora* sp. ("M1") or *Glomus* sp. ("M2"), and
115 by the rhizobial mixture ("S1"), either alone or together.

116 One bean seed was sown in 1-litre mesocosms with 1 kg of a sterile soil-sand mixture
117 placed in a greenhouse with a 12-hour photoperiod and 28/18°C day/night temperature. Tap

118 water was supplied every two days to adjust moisture to nearly 80% of water-holding
119 capacity, i.e. 31 g of water per 100 g of the dry soil-sand mixture. The soils were inoculated
120 on sowing with AM fungi and/or one week after sowing with rhizobia. For AM fungi, the
121 strains were maintained on *Sorghum* sp. grown for 6 weeks in sterilised sand, and a sand
122 inoculum (1 g of chopped AM-colonized sorghum roots (75% infection levels) and 50 g of
123 sand) was placed in a 5-cm slot made near the seedling (Duponnois et al., 2001). For rhizobia,
124 5 ml of liquid inoculum, grown on yeast mannitol broth (YMB) for 24 h, was applied to the
125 base of each seedling. The negative controls (S0 and M0) were managed in the same way as
126 the positive ones, but without inocula, i.e. by adding 5 ml of YMB for S0 or a mixture of sand
127 and non-mycorrhizal sorghum roots for M0.
128 Plants were harvested two months after sowing. Shoots and roots were gently separated
129 and washed to remove the soil, and their biomass was determined after drying at 65°C for
130 48 h. The N and P contents of the shoot biomass were determined by the Kjeldahl method and
131 by colorimetry (molybdenum blue) after acid digestion, respectively. Nodules were separated
132 from fresh roots and counted. Fresh roots were stained with Trypan Blue (Phillips and
133 Hayman 1970) and the percentage of root length colonized by the mycorrhizal fungus was
134 quantified by the Giovannetti and Mosse method (1980) using 30 root segments. Soil
135 phosphatase activity was measured by the Tabatabai method (1994), using the hydrolysis of
136 p-nitrophenylphosphate (p-NPP), buffered at pH 6.0 for acid phosphatase measurement, and
137 pH 11 for alkaline phosphatase measurement, respectively, using a citrate-phosphate buffer
138 (i.e. McIlvain buffer). Means and standard deviations were calculated per treatment for all
139 variables. In order to test the interaction significance between plant mutualists, we performed
140 a two-way ANOVA with AM fungi and rhizobia inoculations as factors, including three
141 treatments for the AM fungus factor (None, M1 and M2) and two treatments for rhizobia (S0,
142 S1). The two-way ANOVAs were followed by Tukey HSD post hoc tests to localize the

significant differences between treatments and display letters of pair-wise comparisons. When the interaction was significant, the Tukey HSD post hoc results from the interaction were displayed. When the interaction was not significant, the Tukey HSD post hoc results for the main effects were displayed. The ANOVA residuals were checked for normality using Wilk-Shapiro tests. All tests were performed using R software (R Core Team, 2015) at $P < 0.05$.

3. Results

After two months of growth, shoot biomass was significantly impacted by the inoculation of rhizobia (*P*-value 0.012) and mycorrhiza (*P*-value 0.021), without any significant interaction (*P*-value 0.251) between these two factors (Table 1). The inoculation of rhizobia (S1) and AM fungus (M1, M2), alone or together, induced higher shoot biomass (around 50% more) than the controls (S0 and M0). The total plant biomass showed similar patterns with the highest values for treatments S1 (0.29 g) and M1 (0.30 g). In contrast, we did not find any significant changes in root biomass or in the shoot:root ratio between the treatments. A slight increase (P-value <0.001) in the total N content of the plant was observed with the inoculation of both rhizobia (+ 13%) and mycorrhiza (+7%). Thus, the amount of N accumulated in plant biomass increased by 70% after inoculation with rhizobia or mycorrhiza. Alkaline phosphatase activity exhibited the same trend as shoot and total plant biomass, i.e. higher values for the inoculation of rhizobia (*P*-value 0.013) and mycorrhiza (*P*-value 0.001), without any significant interaction (P-value = 0.401). AM fungal isolate M2 was more efficient than M1.

The plant P content, the number of nodules, the mycorrhizal rate of the plants and acid phosphatase activity were affected by inoculation with both AM fungi and rhizobia, with significant interactions (Table 1). The plant P content, which was 0.85 g kg^{-1} without inoculation (S0-M0), increased to 1.57 g kg^{-1} with dual inoculation (S1-M2), corresponding to

168 an amount of P accumulated in plant biomass that was three times greater. The nodule
169 number, which was zero in the absence of inoculation with the rhizobium strains, reached 134
170 nodules per plant after inoculation. However, co-inoculation of soil with mycorrhizal strains
171 increased nodulation by 77-89%. The mycorrhization rate, which was also zero in the absence
172 of inoculation, increased slightly (12%) following inoculation with rhizobia. The
173 mycorrhization rate increased from 30-63% for inoculation with mycorrhiza alone to 80-95%
174 for inoculation with both mycorrhiza and rhizobia, i.e. an increase of 28-216% (Table 1).
175

176 **4. Discussion**

177 We showed positive responses of legumes to rhizobial and AM symbioses, as often found
178 (Xie et al., 1995; Ndoye et al., 2015), despite strong N and P depletion in these tropical soils.
179 Based on greenhouse experiments, a meta-analysis showed an increase in yield of 59% for
180 rhizobial inoculation, 45% for AM fungi and 44% for rhizobial and AM fungi (Kaschuk et al.,
181 2010), in line with our results. However, we found a synergistic effect of dual inoculation on
182 plant P content, nodulation, mycorrhizal rate and acid phosphatase activity. Few data have
183 shown a similar synergistic effect of dual inoculation (e.g. Chalk et al., 2006; Ossler et al.,
184 2015), with most results showing that the effects of dual inoculation are only additive (see the
185 quantitative review of Larimer et al. 2010). Synergistic benefits of dual inoculation are
186 thought to occur mostly in soils with both limited N and P availability (Mortimer et al., 2012).
187 According to the stress-gradient hypothesis for plant communities predicting an increasing
188 importance of facilitative mechanisms relative to competition along gradients of increasing
189 environmental stress (Maestre et al., 2009), it is possible that dual inoculation is likely to
190 produce synergistic effects in severely nutrient-depleted Malagasy soils. This hypothesis is
191 supported by the fact that the main mechanism by which inoculation with AM fungi acts on
192 plant functions seems to be improved phosphatase activity, particularly that of acid

193 phosphatases responsible for organic P hydrolysis (Tabatabai, 1994) and involved in
194 supplying the high P requirements of N_2-fixing nodules (Sulieman and Tran, 2015). However,
195 according to Zhang et al. (2016), phosphatase activity would not seem to be due to the AM
196 fungus itself, but to a free-living phosphate-solubilizing bacterium associated with AM fungi.
197 Our study, carried out on a tropical soil with high P-fixing capacity and poor N availability,
198 supported the hypothesis of a contribution of acid phosphatase activity and showed a highly
199 significant positive interaction between the two symbionts on the number of nodules and the
200 mycorrhization rate. However, the costs and benefits associated with these interactions for the
201 plant are context-dependent, with AM fungi and rhizobia being less beneficial to plants in
202 environments high in P (Hoeksema et al., 2010) or N (Herridge et al., 1984) (in Larimer et al.,
203 2014). However, dual symbioses with AM fungus and rhizobia contribute to the success of
204 legumes, especially in a harsh environment and on low-fertility soils (Franco and de Faria,
205 1997; van der Heijden et al., 2016).
206 We also highlighted the positive effect of native symbiotic microorganisms selected from
207 the soils of the Malagasy Highlands. Native microorganisms can display better adaptability to
208 soil and environmental stress under harsh conditions (e.g. Kawaka et al., 2014), with the M2
209 strain displaying greater efficiency. As the quality of the commercial products used as
210 biofertilizers is sometimes questionable (Herrmann and Lesueur, 2013) and the potential
211 negative consequences of introducing microorganisms into the soil is poorly understood
212 (Thomsen and Hart, 2018), commercial inoculants should be used with caution, especially in
213 ecosystems like Madagascar, which are hotspots of endemic biodiversity that need to be
214 protected (Mittermeier et al., 2011).
215

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Variables	**Unit**	**Factors**																	
		Rhizobium inoculation treatments							***Mycorrhiza inoculation treatments***										***Interaction***
		S0			S1			*P*-value	M0			M1			M2			*P*-value	*P*-value
Shoot biomass	g	0.14	(0.05)	**x**	0.21	(0.07)	**y**	**0.012***	0.13	(0.05)	**α**	0.21	(0.04)	**β**	0.20	(0.09)	**αβ**	**0.021***	0.251
Root biomass	g	0.07	(0.03)	x	0.07	(0.04)	x	0.330	0.05	(0.03)	α	0.08	(0.04)	α	0.07	(0.02)	α	0.202	0.661
Total biomass	g	0.23	(0.09)	**x**	0.29	(0.10)	**y**	**0.034***	0.18	(0.09)	**α**	0.30	(0.08)	**β**	0.26	(0.11)	**αβ**	**0.034***	0.521
Shoot:Root	/	2.77	(0.94)	x	3.30	(1.31)	x	0.294	2.98	(0.87)	α	3.09	(1.33)	α	3.09	(1.39)	α	0.982	0.228
Plant N content	g kg^{-1}	18.8	(0.82)	**x**	21.3	(1.05)	**y**	**<0.001*****	19.1	(1.37)	**α**	20.4	(1.15)	**β**	20.6	(1.86)	**β**	**<0.001*****	0.225
Plant P content	g kg^{-1}	1.09	(0.19)		1.33	(0.23)			0.95	(0.12)		1.30	(0.08)		1.38	(0.23)			**0.006****
Alkaline phosphatase	µg-pNP h^{-1} g^{-1}	0.9	(0.8)	**x**	1.5	(0.7)	**y**	**0.013***	0.5	(0.4)	**α**	1.1	(0.6)	**α**	2.0	(0.6)	**β**	**0.001***	0.401
Mycorrhization rate	%	30.9	(29.0)		62.2	(38.8)			40	(42.7)		71.2	(13.6)		67.1	(34.7)			**<0.001*****
Nodule number	Number	0.0	(0.0)		208.3	(59.0)			67.1	(72.3)		118.5	(128.6)		145.0	(136.0)			**<0.001*****
Fluorescein diacetate	µg-FDA h^{-1} g^{-1}	179.1	(25.6)		171.5	(108.8)			104.1	(80.2)		197.6	(49.9)		224.3	(39.3)			**<0.001*****
Acid phosphatase	µg-pNP h^{-1} g^{-1}	2.8	(3.0)		5.0	(3.3)			0.91	(1.1)		5.0	(2.6)		5.8	(3.4)			**0.036***
Significant interaction		**Treatments**																	
		S0-M0			S1-M0			S0-M1			S1-M1			S0-M2			S1-M2		
Plant P content	g kg^{-1}	0.85	(0.02)	**d**	1.05	(0.07)	**bc**	1.24	(0.06)	**c**	1.36	(0.05)	**ab**	1.17	(0.12)	**cd**	1.57	(0.08)	**a**
Mycorrhization rate	%	0.0	(0.0)	**d**	11.5	(14.4)	**cd**	62.5	(14.2)	**b**	80.0	(4.8)	**ab**	30.0	(2.0)	**c**	95.0	(1.6)	**a**
Nodule number	Number	0.0	(0.0)	**c**	134.2	(14.0)	**b**	0.0	(0.0)	**c**	253.7	(15.4)	**a**	0.0	(0.0)	**a**	237.0	(34.6)	**a**
Fluorescein diacetate	µg-FDA h^{-1} g^{-1}	175.8	(18.4)	**ab**	32.3	(18.2)	**c**	168.7	(38.9)	**b**	226.5	(46.9)	**a**	192.96	(17.4)	**ab**	255.7	(24.6)	**a**
Acid phosphatase	µg-pNP h^{-1} g^{-1}	0.06	(0.07)	**bc**	1.76	(1.09)	**bc**	5.49	(3.44)	**ab**	4.55	(2.34)	**abc**	3.01	(1.68)	**bc**	8.77	(1.30)	**a**

When the interaction was not significant, letters "x, y" and "α, β" indicate main effect significance within rhizobium and mycorrhiza treatments, respectively. When the interaction was significant, letters "a, b, c, d and e" indicate significant difference among cross-treatments according to Tukey HSD test ($P < 0.05$, n=5).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$