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Formononetin stimulates mycorrhizal fungi colonization on the surface of active root nodules in soybean

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Abstract The symbiosis of Leguminosae with arbuscular mycorrhizal fungi (AMF) and N_2-fixing nodulating bacteria (NFNB) can occur simultaneously, forming a tripartite symbiosis. In particular, AMF can colonize root nodules, although this interaction is not yet well elucidated, especially with regard to nodule activity and to the influence of external factors, such as biostimulants. In this study, we hypothesized that the application of the flavonoid formononetin, used to stimulate root colonization by native AMF, increases the AMF colonization of soybean (*Glycine max*) root nodules, especially under low availability of phosphorus (P). To test this hypothesis, we performed a field experiment in randomized blocks in a 4 × 3 factorial design, with 4 treatments of formononetin (0, 0.46, 0.92 and 1.84 g per kg seed) and 3 of P (0, 60 and 120 kg ha^{-1}) with 5 replicates. Nodules and roots were collected during the R2 stage (full flowering) and evaluated with respect to AMF colonization. Formononetin stimulated mycorrhizal fungi colonization of active nodules, especially when no P was applied, as also observed for AMF root colonization; however, it had no effect with 60 and 120 kg P ha^{-1}. Thus, the application of formononetin increases surface AMF colonization of active nodules and roots, but its effect disappears with an increase in P and the inactivity of the nodule.

Keywords *Glycine max* · Biostimulant · Nodule colonization · Root symbiosis · Nitrogen fixation · Mycorrhiza

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1 Introduction

Leguminosae plants can establish tripartite symbioses with arbuscular mycorrhizal fungi (AMF) and N_2-fixing nodulating bacteria (NFNB). Through their hyphae, AMF broaden the soil exploration area of the roots, increasing the absorption of nutrients that are less mobile in the soil, especially phosphorus (P) (Smith and Read 2008). The bacteria, in turn, provide N that is accessible to the plant. The simultaneous occurrence of both symbioses can have synergistic, neutral or antagonists effects on plant growth, depending on the identity of the symbionts (Azcón et al. 1991; Ahmad 1995; Xavier and Germida 2002; Franzini et al. 2010; Xiao et al. 2010). The factors that determine the functional compatibility in the tripartite symbiosis are not yet understood, with both nutritional (e.g. enhanced fitness of the common host plant due to increased nutrient uptake, specially P by AMF and nitrogen by symbiotic N_2 fixation but also a possible competition for host photosyntates) (Mortimer et al. 2008; Kaschuk et al. 2009) and non-nutritional (production of signaling molecules that elicitate root infection by both symbionts) interactions (Xie et al. 1995; Catford et al. 2003, 2006) being reported. Besides, it is possible that nodule colonization by AMF plays a role in the compatibility among those symbionts (Vidal-Dominguez et al. 1994; Scheublin and van der Heijden 2006). However, this interaction is yet poorly understood.

Root nodules are highly specialized structures with many specific physiological and anatomical specific features that

make them unique when compared to other parts of the root (Udvardi and Poole 2013). However, they can be colonized by AMF as demonstrated by microscopy techniques in *Phaseolus vulgaris* (Baird and Caruso 1994), *Trifolium repens*, and *Medicago sativa* (Vidal-Dominguez et al. 1994) and in three species of Leguminosae (*Lotus corniculatus*, *Trifolium repens*, and *Ononis repens*) naturally found in dunes (Scheublin et al. 2004). If nodule colonization by AMF could direct supply P to the nodule, the tripartite symbiosis would increase nitrogen fixation because this process requires large quantities of P, as shown by Vadez et al. (1997) who reported that P concentration in the nodule is usually about three times higher than that found in other parts of the root.

Although inoculation with NFNB strains is an established and widely used technique in agricultural systems (Moreira and Siqueira 2006), the agricultural application of mycorrhizal symbiosis is limited, mainly due to the difficulty in producing inoculants (Powell and Bagyaraj 1984; Douds et al. 2006) because of the obligatory symbiotrophy of AMF, and because little is known regarding the performance of different AMF isolates under varying edaphic and climatic conditions. Thus, alternative techniques have been developed, such as those based on the stimulation of root colonization by AMF native to soil through the application of biostimulants, which are signaling molecules that play a natural role in the communication between the plant and AMF during the colonization process and whose primary example is formononetin (Nair et al. 1991; Siqueira et al. 1991).

This isoflavonoid can stimulate root colonization by AMF, especially under low P availability (Davies et al. 2005a, b). If AMF nodule colonization is controlled by the same factors that operate in AMF root colonization, it is possible that formononetin also increases AMF nodule colonization and that its effect is dependent on P availability to the plant. Therefore, we hypothesized that the application of the flavonoid formononetin on soybean (*Glycine max*) increases the AMF colonization of root nodules, especially under low availability of phosphorus (P).

2 Material and methods

2.1 Study area characterization

A field experiment was performed from December 2010 to April 2011 at an experimental farm located in the southeast of Minas Gerais, Brazil, at 21°12’17” S, 44°58’49” W, with an altitude of 957 m and an annual average rainfall of 1411 mm. The area where the experiment was performed was kept as fallow since 2009, then it was used for maize production until April 2010. From April to December 2010, the area was again kept in fallow with the growth of spontaneous vegetation, predominantly *Brachiaria* sp. Before 2009, this area has been used mainly for the production of maize and soybean. The soil of the area is classified as Red-Yellow Latosol (Oxisol according USDA classification), and its chemical and physical characteristics were the following: pH in $H_2O = 6.50$; $H + Al = 2.3$ $cmol_c\ dm^{-3}$; Al = 0.0 $cmol_c\ dm^{-3}$; Ca = 3.8 $cmol_c\ dm^{-3}$; Mg = 1.5 $cmol_c\ dm^{-3}$; CTC = 7.9 $cmol_c\ dm^{-3}$; K = 0.26 mg dm^{-3}; *P* = 10.3 mg dm^{-3}; soil organic matter =27 g kg^{-1}; clay =570 g kg^{-1}; silt =80 g kg^{-1}; sand =350 g kg^{-1}. Soil pH was measured in a soil/H_2O suspension (1:2.5 *w*/*v*). Exchangeable Al, Ca and Mg were extracted with 1 mol l^{-1} KCl solution. Al was measured by titration and both Ca and Mg by atomic-absorption spectrophotometry. Available potassium and phosphorus were extracted with Mehlich I solution (Mehlich 1953). Then, K was determined by flame photometry and P by colorimetry. Organic carbon (Corg) was determined by titration with a solution of ferrous ammonium sulfate after oxidation of the carbon by potassium dichromate (Walkley and Black 1934). The soil texture was determined by using the hydrometer method (Bouyoucos 1951).

2.2 Experimental design

The experimental design was randomized blocks with two crossed factors: 3 treatments of phosphorus (0, 60, and 120 kg ha^{-1} of P_2O_5, the last two corresponding to 50 and 100 % of the recommendation based on soil analysis); and 4 treatments of the biostimulant formononetin (Myconate®, Plant Health Care Inc.) (0, 0.46, 0.92 and 1.84 g per kg seed, corresponding to 0, 25, 50, and 100 % of the recommend by the manufacturer), with 5 replicates for a total of 60 plots. Each plot had 6 rows with 10 m spaced by 0.45 m.

Soil preparation was performed with a plowing and a harrowing cycle followed by the opening of furrows. Fertilization was performed manually before sowing by applying P_2O_5 corresponding to each treatment along with 40 kg ha^{-1} of K_2O in the furrows. The variety of soybean used was FAVORITA RR (provided by the Departamento de Agricultura, Universidade Federal de Lavras), which is recommended for the region. The seeds were inoculated with a commercial peat inoculant containing *Bradyrhizobium japonicum* (SEMIA 5079 and 5080), in the proportion 100 g of inoculant (5×10^9 cells g^{-1}) per 50 kg of seed. Sowing was manually performed in December 2010 by uniformly distributing 15 seeds per linear meter, and the harvest was performed in April 2011. Culture treatments were performed uniformly on all experimental plots according to the need of the culture.

2.3 Collection of nodules and roots

During the R2 stage, indicated by the full flowering of soybean plants, random collection of nodules and roots was performed on three plants from each of the 60 plots by carefully taking a volume of approximately 1 dm^3 of soil with the root system from each plant. Nodules attached to the root or in the

soil were manually collected. The R2 stage was chosen because it is when the peak of nitrogenase activity in nodules occurs and it also coincides with the peak of nutrients demand by plants. From the total number of nodules collected, fifteen nodules from each of the three plants were randomly pooled in order to obtain a total of 45 nodules per plot.

Nodules were removed from roots, washed in tap water, and cross-sectioned. Immediately after sectioning, they were classified according to their activity by analysis of their inner color, which varies according to the presence of leghemoglobin (Minchin et al. 2008). Nodules were considered active only when their internal color was clearly reddish, with larger proportion of magenta than the 0/60/20/0 color on the % scale cyan/magenta/yellow/black (CYMK), compared to a gradient of colors in a printed color chart in the CYMK scale under the same light source. Nodules with a lower proportion of magenta and a larger proportion of yellow than the 0/40/40/0 color on the same scale were classified as inactive. Nodules with intermediate colors were discarded to avoid ambiguity in the classification.

Five other plants were also collected per treatment as above to sample fine roots for assessing the percentage of AMF colonization. The fine roots were randomly taken from each plant and pooled to form a composite sample for each plot.

2.4 Evaluation of surface colonization of active and inactive nodules and the percentage of AMF colonization of soybean roots with different treatments of P and formononetin

Nodules selected as previously described were heated for 1 h in KOH (5 %) for clearing, washed in distilled water, immersed in HCl (5 %) for 1 h at room temperature, and then immersed in acid fuchsin for 1 h at 90 °C. Permanent slides were then mounted with nodule sections for microscopic observation. The sections were placed on microscope slides on a solution of polyvinyl-lacto-glycerol (PVLG) and gently crushed with a coverslip. A total of 1200 nodules collected from 60 plots were used to prepare 240 slides with 5 nodules in each; for each plot, a total of 4 slides were prepared, with 2 slides containing active nodules and 2 slides containing inactive nodules. Once prepared, the slides were examined under an optical microscope (Nikon Labophot) for the identification of fungal structures such as hyphae, spores, and vesicles, which are indicative of AMF colonization. The analysis was performed by scanning the entire surface of all nodule sections. Hyphae from AMF were distinguished from other hyphae by the presence of entry points and by the criteria defined by Steinberg and Rillig (2003). The slides were photographed with a NIKON EFD-3 optical microscope coupled to a Canon A630 digital camera. Nodule colonization was calculated for each plot as the proportion of nodules where AMF structures were detected.

Plant roots were depigmented with KOH (10 %) and colored with trypan blue according to the method of Phillips and Hayman (1970) modified by Koske and Gemma (1989), and the percentage of root length colonized was assessed according to the method used by Giovannetti and Mosse (1980).

For scanning electron microscopy analysis, six sections of nodules classified as active or inactive were randomly chosen. The sections were removed from the modified Karnovsky fixing solution (2.5 % glutaraldehyde, 2.0 % paraformaldehyde, 0.05 M cacodylate buffer, pH 7.2) in which they were immersed, transferred to cryo-protecting solution (30 % glycerol) for 30 min, and cross-sectioned in liquid nitrogen. The fragments were transferred to a sufficient amount of 1 % osmium tetroxide solution (3 drops) and water to cover them for an hour, washed three times in distilled water, subsequently dehydrated in an acetone series (25, 50, 75, 90, and 100 %, each three times), and taken to the critical point apparatus. Specimens were mounted on aluminum stubs, covered with gold, and observed under a LEO EVO 40 XVP scanning electron microscope, where images were generated for analysis.

2.5 Statistical analysis

Data exploration following the protocol described in Zuur et al. (2010) was performed before the analysis. For the proportion of nodules colonized by AMF, a generalized linear mixed-effects model was used with a binomial distribution in which the proportion of nodules colonized (dependent variable) was modeled by the P, formononetin, and the activity of nodules (independent variables) and their interactions (fixed effects). Due to the block design and because nodule activity was nested within the combinations of P and formononetin treatments, random intercepts for blocks and for the main plots were used. For comparing alternative models, likelihood ratio tests were performed to select the best model following Zuur et al. (2009).

For the AMF colonization of roots, a generalized linear model with a binomial distribution was used, with the percentage of AMF colonization as the dependent variable and P and formononetin treatments as independent variables. Due to the overdispersion detected in this model, the “quasi” correction was applied. The alternative models were compared by likelihood ratio tests as described by Zuur et al. (2009). Data were analyzed using the lme4 package on R 2.15 (R Development Core Team 2012).

3 Results and discussion

3.1 Structural aspects of soybean nodules colonized by mycorrhizal fungi through photomicrographs and optical scanning electron microscopy

Surface colonization of nodules by AMF occurred in both active and inactive nodules, as observed by the presence of cenocytic hyphae (aseptate), spores (except for active

nodules), and vesicles (Fig. 1). Because nodule colonization was evaluated by using crushed nodules, the cases when the nodules are only surface colonized or when both surface and internal colonization occurred could not be clearly distinguished, thus the term surface colonization is used herein. Appressorium-like structures were common in both active and inactive nodules, being characterized by the presence of a swelled hypha on nodule surface (Fig. 1a). The occurrence of appressoria on the surface of active nodules indicates internal AMF colonization in these nodules, although these structures can be occasionally aborted without penetrating the roots (Giovannetti et al. 1993). With regard to the fungal structures found, hyphae predominated in both active and inactive nodules, with a minor presence of vesicles (Fig. 1b), whereas spores only occurred in inactive nodules (Fig. 1c). There were no apparent differences in the shape and size of vesicles corresponding to the activity of the nodule. Spores and vesicles were present in an insignificant amount (less than 0.1 % of the total nodules analyzed, 1200). This low sporulation in the nodules differs from the results reported by Vidal-Dominguez et al. (1994) by studying *Glomus fasciculatum* in two Leguminosae species (*Trifolium repens* and *Medicago sativa*). Differences in the legume species as well as experimental conditions could be responsible for the discrepancy between our results and those of Vidal-Dominguez et al. (1994).

Arbuscules were not observed in colonized nodules, although they were abundantly present in roots. With the exception of Baird and Caruso (1994), who reported the occurrence of structures similar to degenerated arbuscules in common bean nodules, there are no other reports on the occurrence of arbuscules in nodules.

Table 1 Selected models for AMF colonization of nodules and roots of soybean under doses of P and formononetin

	Parameter	Estimate	Standard Error	*P*
Nodules[a]	Intercept	−1.7407	0.2432	< 0.001
	Nodule activity (inactive)	−0.1192	0.0774	0.123
	P	0.0016	0.0031	0.602
	Formononetin	0.5084	0.2264	0.023
	Nodule Activity (inactive): Formononetin	−0.6560	0.0799	< 0.001
	P: Formononetin	−0.0070	0.0029	0.017
Roots[b]	Intercept	0.2267	0.1150	0.054
	P	0.0002	0.0011	0.818
	Formononetin	0.2435	0.0860	0.006
	P: Formononetin	−0.0023	0.0011	0.042

[a] Akaike information criteria (AIC) = 133.5

[b] quasi-likelihood AIC = 56

3.2 Evaluation of surface colonization of active and inactive nodules and the percentage of AMF colonization of soybean roots after treatment with different treatments of P and formononetin

The selected models for AMF colonization of roots and nodules are shown in Table 1. The response of AMF colonization to formononetin and P indicated similar trends in roots and

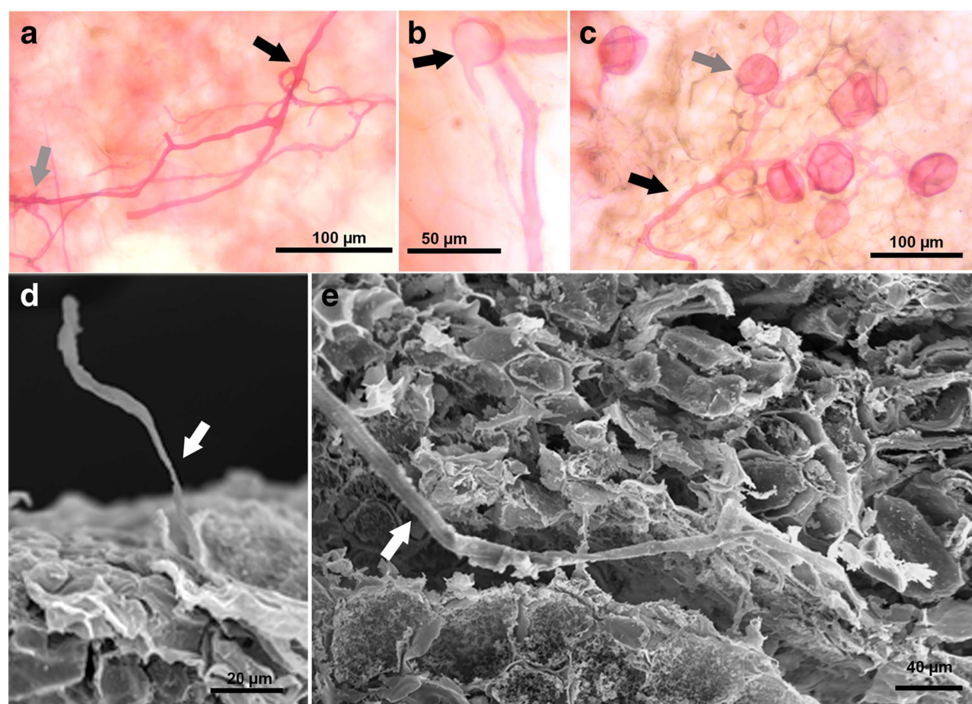

Fig. 1 Optical and scanning electron microscopy images of *Glycine max* (L.) root nodules colonized by arbuscular mycorrhizal fungi**. a**) Surface of active nodule showing fungal hyphae (*black arrows*) and entry point (*gray arrow*); **b**) Vesicle (*black arrow*) in an active nodule; **c**) Surface of inactive nodule with fungal hyphae (*black arrows*) and spores (*gray arrow*); **d**) Surface of active nodule showing fungal hyphae (*white arrows*); **e**) Cross section showing inactive nodule with fungal hyphae (*white arrows*)

active nodules, as shown in Fig. 2. In both cases, there was a negative interaction between treatments of P and formononetin on AMF colonization of roots and nodules (Table 1). Without P, the estimated coefficients for formononetin were positive and significant at the 5 % level for both roots and active nodules (Fig. 3), indicating that AMF colonization increased linearly with increasing doses of formononetin within the studied interval when no P fertilizer was applied. At 60 and 120 kg P ha^{-1}, the estimated coefficients for formononetin were non-significant at the 5 % level

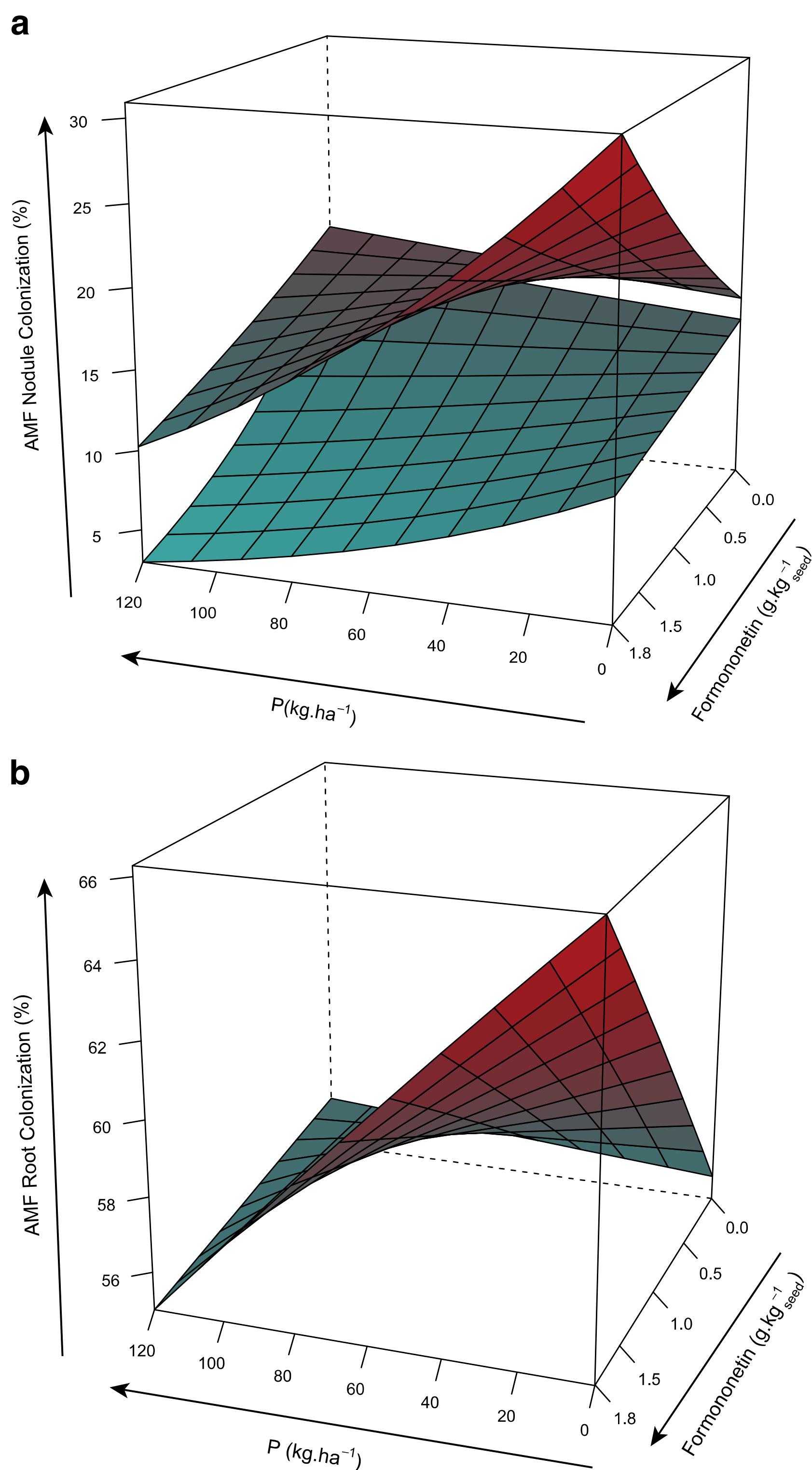

Fig. 2 Mycorrhizal colonization of roots and nodules. **a**) Mycorrhizal colonization of *Glycine max* (L.) active (*top surface*) and inactive (*bottom surface*) nodules with different treatments of P and formononetin; **b**) Mycorrhizal colonization of roots with different treatments of P and formononetin

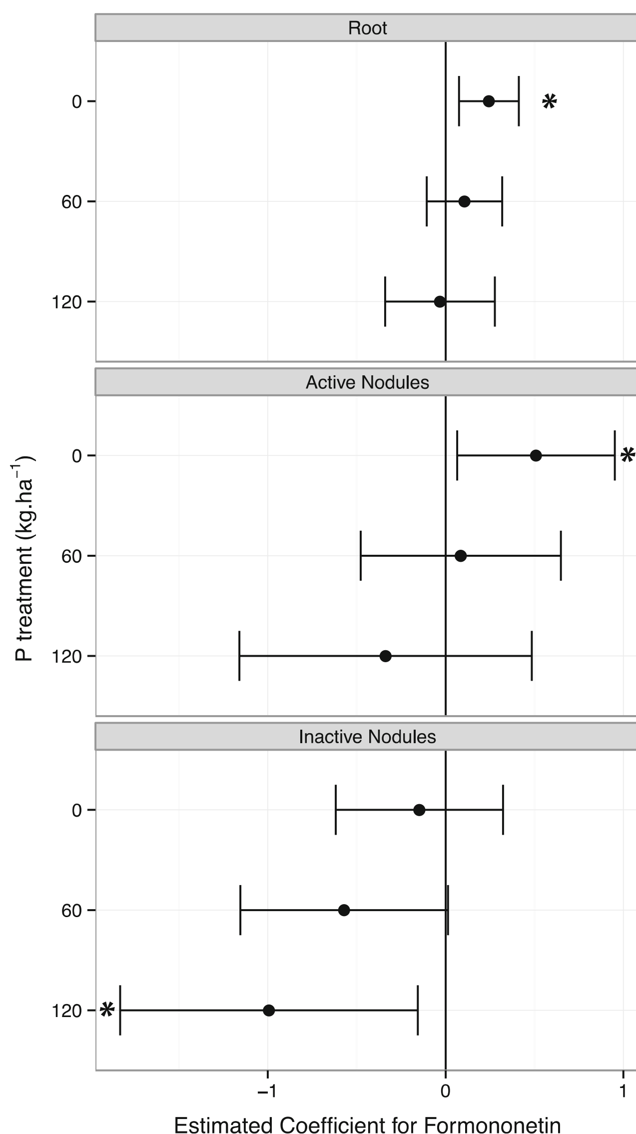

Fig. 3 Coefficients for the effect of formononetin on AMF colonization derived from the models shown in Table 1. The *horizontal bars* indicates the 95 % confidence interval. Coefficients are not statistically significant when their confidence interval includes zero (*vertical line*)

for roots and active nodules. For inactive nodules, the effect of formononetin on AMF colonization was negative and significant at the 5 % level at 120 kg P ha^{-1}. Fitted values for all models for the combination phosphorus and formononetin are presented in Table S1.

The positive effect of formononetin on the AMF colonization of roots has been widely reported (Siqueira et al. 1991; Davies et al. 2005a, b; Antunes et al. 2006; Catford et al. 2006; Novais and Siqueira 2009); however, this is the first report to describe the effect of this isoflavonoid on the colonization of nodules. The mechanisms of the effect of formononetin on root AMF colonization were not yet elucidated, but it is known that exogenous application of formononetin can reduce peroxidase activity (Fries et al. 1998) and induce the catalase activity (Lambais et al. 2003), which are enzymes associated with the control of plant defense during AMF root colonization. Formononetin also directly stimulates AMF spores germination and hyphae growth (Nair et al. 1991).

According to Vidal-Dominguez et al. (1994), nodules growing in non-mycorrhizal roots can be directly colonized by external hyphae. Consequently, nodule colonization does not only occur from the internal root mycelium. For that reason, formononetin may stimulate AMF colonization of the nodule in a manner similar to its action on root. Furthermore, the increase in AMF colonization of the roots caused by the formononetin increases the likelihood of colonization of nodules by hyphae originated from colonized roots.

The similarity between the increasing rates of AMF colonization in response to decreasing dosages of P and increasing doses of formononetin in roots and active nodules may likewise be explained; it has been widely reported that with high availability of P, the plant negatively regulates AMF colonization of roots (Siqueira et al. 1984; Siqueira and Colozzi-Filho 1986; Sena et al. 2004; Nogueira and Cardoso 2006, 2007; Sheng et al. 2012) even in the presence of biostimulants (Rodríguez and Gómez 2011).

Our results reopen the discussion of whether or not the colonization of active nodules by AMF directly affects nitrogen fixation. This subject has not been addressed since 2006. This interaction could be synergistic, neutral or competitive. The fact that we did not observe arbuscules in the colonized nodules supports the hypothesis that this interaction is neutral. In addition, the fact that nodules remain active after colonized by AMF supports the neutrality of the interaction.

4 Conclusions

Increasing doses of formononetin linearly increase the colonization of nodules and roots of *Glycine max* by AMF when no P was applied; however, this effect is reduced or eliminated by 60 and 120 kg P ha^{-1}, respectively, or when nodules are inactive. Soybean active nodules under field conditions were more colonized by AMF than inactive nodules**.**

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