

Seed inoculation with arbuscular mycorrhizal fungi propagules enhanced yield, biomass accumulation, and plant vigor of soybeans (*Glycine max*) and maize (*Zea mays*)

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Abstract

Hyphal networks, resulting from mutualistic symbiotic associations between plants and arbuscular mycorrhizal fungi (AMF), improve plant growth and nutrition by enhancing root access to a significantly larger soil surface area, which increases nutrient availability and translocation within the plant-host. Considering the limitations imposed by the low nutrient availability commonly found in the highly weathered soils of Brazil, the positive effects of AMF could benefit the production of important plant species, such as soybeans and maize. Multiple trials were thus carried out to investigate growth and yield responses of two major crops of Brazil, soybeans and maize, in response to mycorrhizal symbiosis originated from the inoculation of a mixture of four AMF species at seed treatment. Five soybean and seven maize field trials were conducted in the 2018/2019 growing season in different regions of Brazil. Two product rates (10 or 20 ml ha⁻¹) were tested under two P fertilization levels (0% and 100%). We observed that plant growth-promoting effects were observed in response to AMF inoculation in both crops and both rates (10 and 20 ml ha⁻¹ and regardless of P fertilization). Moreover, treatments inoculated with AMF propagule, but not mineral fertilized with P, presented growth improvements either similar or better than the untreated P-fertilized control. Both crops yield was increased when the highest product rate was applied in combination with P fertilization relative to the P-fertilized untreated control. Our results show that EndoFuse is an alternative to facilitate important crops cultivation.

Keywords: symbiosis; crop productivity; phosphorus; *Rhizophagus irregularis*; *Claroideoglomus luteum*; *Claroideoglomus etunicatum*.

Abbreviations: AMF_arbuscular mycorrhizal fungi; DAS_days after sowing; DAB_dry aboveground biomass; TGW_thousand grain weight.

Introduction

The maintenance of the population of beneficial organisms in the soil through sustainable farming practices has gained increased attention in the past decades (Thirkell et al., 2017). It has been reported that the associated benefits provided by higher soil fungal hyphae density in the soil can significantly improve both water and nutrients available to the plants (Paul et al., 2013; Cavagnaro, 2016; Thirkell et al., 2017). Under tropical conditions, such agricultural practices could be of great importance, considering that these region's soils commonly display low availability of mineral nutrients due to the highly advanced pedogenetic developmental stages (e.g. highly weathered soils) (Allen and Hajek, 1989). Such highly weathered soils presenting low nutrient availability are commonly found in both soybean (*Glycine max* (L.) Merr.) and maize (*Zea mays* L.) producing sites of Brazil, one of the most

important grain producers of the world (Lepsch, 2002; Fink et al., 2014). Indeed, the availability of essential nutrients, especially P, can be reduced as a result of intense adsorption of this nutrient into soil colloids under acidic conditions, which can severely harm soybeans and maize growth due to high exchangeable Al³⁺ concentrations (Embrapa, 1997).

Given the economic importance of both soybean and corn production to meet the world's food demands, the use of symbiotic beneficial microorganisms, such as mycorrhizas, could be an important alternative to alleviate the negative impacts of weathered soils features on these crops' productions (Thirkell et al., 2017). Indeed, it has been shown that the association between plants and obligate biotrophic fungi of the phylum Glomeromycota (arbuscular mycorrhizal fungi - AMF) can significantly increase nutrient uptake from the

soil and improve growth (López-Pedrosa et al., 2006; Birhane et al., 2012; Smith and Smith, 2012; Ahanger et al., 2014; Rouphael et al., 2015; Bowles et al., 2016) by increasing the area of soil explored by the root-system (Parniske, 2008; Bonfante and Genre, 2010). It has been also shown that AMF induces resistance to a variety of abiotic stresses, such as salinity and drought (Rodríguez et al., 2008; Ahanger et al., 2014; Salam et al., 2017). Moreover, AMF of Glomeromycota phylum can form intra-cellular symbioses with several important food crops (Smith and Read, 2008), which facilitates their up-scale use in sustainable agricultural systems.

Nonetheless, the beneficial effects mediated by AMF on plant growth and nutrient uptake depend on environmental conditions, as well as on nutrient availability (Smith et al., 2009; Johnson et al. 2015). Thereby, despite a large inherent potential arising from the utilization of AMF as soil conditioners, determination of the response of these microorganisms under different nutrient availability and conditions and their best propagule concentration and viability is of great importance. Multiple experiments were carried out to investigate growth and yield responses of two major crops of Brazil, soybeans and maize, in response to mycorrhizal symbiosis originated from the inoculation of a mixture of four AMF species at seed treatment. We hypothesized that treating soybeans and maize seeds with AMF propagules will positively increase plant growth and yields, especially under conditions of low P availability.

Results and discussion

Supplementary Table S1 presents climatic conditions and other key information of five soybeans and seven maize field trials designed to study AMF-enrichment effects onto these crops, respectively. Soybean and maize-growing sites presented edaphoclimatic conditions to which such crops are commonly exposed in Brazil, as well as displayed P levels in the soil (Supplementary Table S1 and Supplementary Figure S1). The list of treatments can be observed in Table 1. Moreover, information about cultivar, sowing dates, target plant density, and row spacing is found in Table 2.

Soybeans trials

ANOVA results indicated that treatments significantly affected soybean yields, thousand-grain weight (TGW), plant height, and vigor at 5% probability ($p < 0.05$) (Supplementary Table S2), whereas treatments effects on dry aboveground biomass (DAB) were only significant at the significance level of 10% ($p = 0.077$, Supplementary Table S2). Furthermore, treatment effects on plant vigor and TGW varied significantly across locations (Supplementary Table S2). Since a significant *Treatment x Location* interaction indicates that treatment effects varied according to the experimental site, plant vigor, and TGW were also analyzed separately at each experimental site (Supplementary Table S2). Conversely, data from other variables found to be impacted by treatments (i.e. plant height, yields, and DAB) were pooled for discussion, as such were not significantly affected by the experimental site ($p > 0.05$).

All variables assessed at the present study were positively impacted by arbuscular mycorrhizal fungi (AMF) propagule inoculation as a seed treatment (Table 3), particularly when

results are compared to untreated control treatments within each category (e.g., with or without P fertilization). Even in the absence of P fertilization, plant heights, vigor, DBA, TGW, and soybean yields at AMF-inoculated treatments matched or outperformed the untreated control P-fertilized counterpart (Table 3).

Results indicated a general trend toward slightly taller and more vigorous soybean plants (Table 3) treated with EndoFuse. Plant vigor and yields displayed the largest response to AMF propagule seed inoculation among all variables (Table 3). When P was added during sowing, both product rates applied at the present work (10 and 20 mL ha⁻¹) enhanced soybean yields significantly, increasing productivities by 4.3% and 7.7%, respectively, compared to the untreated control counterpart (with P fertilization) (Table 3). Even when P fertilizer was not applied, yields increased by 3.4% and 8.3% for each product rate in comparison to the untreated control treatment without P (Table 3), suggesting that AMF inoculation could enhance plants' nutrient uptake capacity, as previously reported (Smith and Smith, 2012; Ahanger et al., 2014; Rouphael et al., 2015; Bowles et al., 2016). Interestingly, in contradiction with our hypothesis, our results demonstrate that yield can be improved in both P scenarios and suggest that AMF seed inoculation can be recommended regardless of P fertilization during crop sowing.

Plant vigor and TGW were also analyzed separately according to data collected in each experimental site, given that treatment effects on these variables varied across locations (as indicated by a significant *Treatment x Location* interaction for those variables on Supplementary Table S2). Two out of five locations were found to display significant differences in TGW among treatments (Fig 1). Importantly, a statistically significant increase in plant vigor was observed at all sites, while the actual intensity (or percentage increase) on these parameters set forth by treatments varied across locations. The largest increases in plant vigor were noticed at Uberlândia/MG, Lavras/MG, and Planaltina/DF – sites with the lowest soil P values in this study (Supplementary Table S1). The more pronounced yield increases reported in experimental sites with lower P availability can be related to the benefits arising from functional AMF symbiosis, since the resulting larger soil surface and enhanced P absorption (Bucher, 2007; Guether et al., 2009; Vergara et al. 2018) would be expected to play a major role in supplying P to crops to offset natural low P availability conditions.

Maize trials

ANOVA results indicated treatments significantly affected plant height, vigor, and maize yields at the 5% level ($p < 0.05$) (Supplementary Table S3), whereas no significant effects were noticed on either DAB or TGW ($p > 0.05$). Treatment effects on plant vigor, DBA, and plant heights varied across locations as indicated by the significant *Treatment x Location* interaction (Supplementary Table S3). Therefore, plant vigor and height, as well as maize DBA values were analyzed separately according to each location. Conversely, maize yields and TGW data were pooled for discussion, as these were not significantly affected by location ($p > 0.05$).

Similar to soybeans, results indicated a general trend toward slightly taller and more vigorous plants resulting from EndoFuse seed inoculation (Table 4). Interestingly, average

Treatment ID	Treatments	P fertilization	Application type
1. Untreated control - P	No AMF ¹ and no P added (-AMF; -P)	No	--
2. EndoFuse ² (10 ml ha ⁻¹) - P	Low rate AMF mixture (0.5x AMF; -P)	No	ST ³
3. EndoFuse (20 ml ha ⁻¹) - P	High rate AMF mixture (1x AMF; -P)	No	ST
4. Untreated control + P	No AMF; only P added (-AMF; +P)	Yes	--
5. EndoFuse (10 ml ha ⁻¹) + P	Low rate AMF mixture with P added (0.5x AMF; +P)	Yes	ST
6. EndoFuse (20 ml ha ⁻¹) + P	High rate AMF mixture with P added (1x AMF; +P)	Yes	ST

¹Arbuscular mycorrhizal fungi; ²Product consisting of a concentrated solution containing 22,500 AMF propagules ml⁻¹; ³Seed treatment.

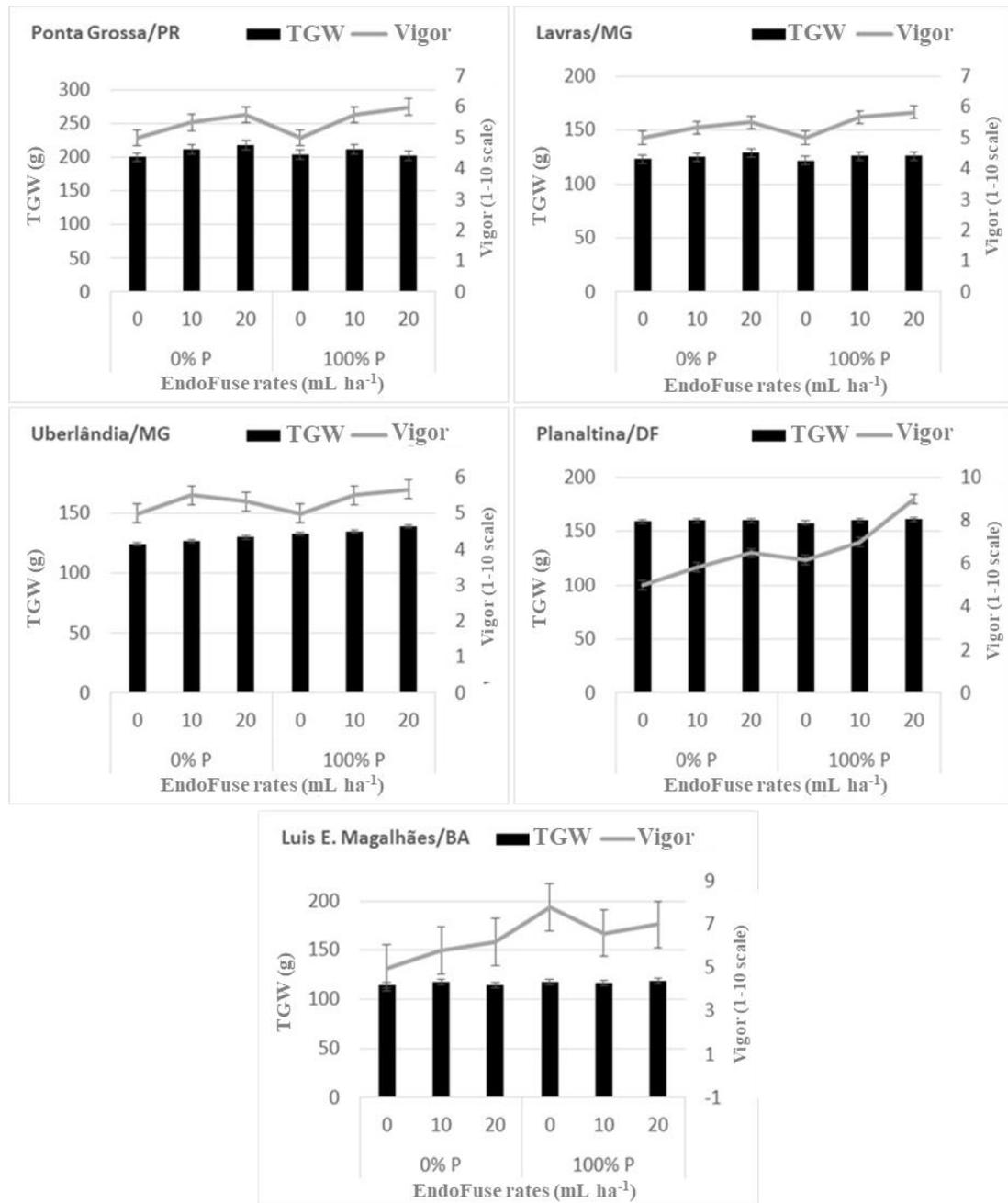


Fig 1. The means values of soybean thousand-grain weight (TGW) and plant vigor in each experimental site. Error bars represent confidence intervals. Non-overlapping confidence intervals indicate significant differences among treatments according to Fisher's LSD test ($p \leq 0.05$).

Table 2. Cultivar, sowing dates, target plant density, and row spacing across five soybeans and seven maize field trials.

Soybeans				
Trial ID (City/State)	Soybean cultivar	Sowing date ¹	Target plant density (plants ha ⁻¹)	Row spacing (m)
1. Ponta Grossa/PR	NA 5909 RG	14/12/2018	300,000	0.50
2. Uberlândia/MG	RK 6719 IPRO2	15/01/2019	300,000	0.50
3. Lavras/MG	97R22 IPRO	19/02/2019	300,000	0.50
4. Planaltina/DF	BMX Bônus IPRO	08/01/2019	100,000	0.50
5. L.E.M./BA ²	MSOY 8349 IPRO	28/12/2018	260,000	0.50
Maize				
Trial ID (City/State)	Maize hybrid	Sowing date	Target plant density (plants ha ⁻¹)	Row spacing (m)
1. Itaara/RS	27D28	11/02/2019	64,000	0.50
2. Ponta Grossa/PR	2A401PW	15/01/2019	90,000	0.50
3. Iperó/SP	30F53R	28/01/2019	160,000	0.50
4. Uberlândia/MG	RK 9110 PRO 2	15/01/2019	80,000	0.50
5. Lavras/MG	DKB 177	30/04/2019	93,331	0.75
6. Planaltina/DF	P3898	08/01/2019	80,000	0.50
7. L.E.M./BA	30F53 VYHR	28/12/2018	80,000	0.50

¹Calendar dates are presented as day/month/year; ²Luis Eduardo Magalhães/BA.

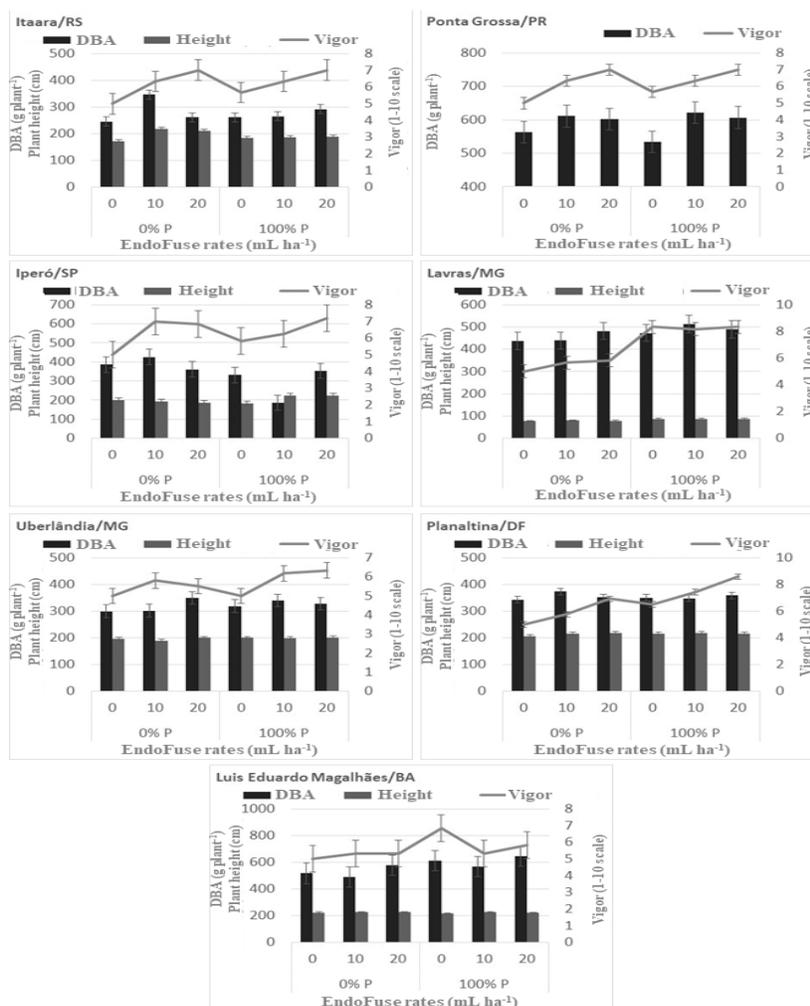


Fig 2. Maize dry aboveground biomass (DBA), plant heights, and plant vigor means in each experimental site. Error bars represent confidence intervals. Non-overlapping confidence intervals indicate significant differences across treatments according to Fisher's LSD test ($p < 0.05$).

Table 3. Average values and mean separation results of dependent variables [plant heights, vigor, dry aboveground biomass (DBA), thousand-grain weight (TGW), and yields] gathered from five soybean field trials.

Treatment	Height ¹ (cm)	Vigor ² (1-10 scale)	DBA ³ (g/5 plants)
1. Untreated control - P	44.9	c ⁶	72.2
2. EndoFuse (10 mL ha ⁻¹) - P	45.7	bc	78.4
3. EndoFuse (20 mL ha ⁻¹) - P	46.7	ab	81.2
4. Untreated control + P	46.7	ab	86.9
5. EndoFuse (10 mL ha ⁻¹) + P	46.8	ab	85.1
6. EndoFuse (20 mL ha ⁻¹) + P	48.2	a	86.3

Treatment	TGW ⁴ (g)	Soybean Yields (kg ha ⁻¹)	Efficiency ⁵ (%)
1. Untreated control - P	141	2587	--
2. EndoFuse (10 mL ha ⁻¹) - P	145	2802	+8.3
3. EndoFuse (20 mL ha ⁻¹) - P	146	2675	+3.4
4. Untreated control + P	144	2676	--
5. EndoFuse (10 mL ha ⁻¹) + P	147	2792	+4.3
6. EndoFuse (20 mL ha ⁻¹) + P	147	2884	+7.7

¹Plant height data were collected at early flowering stages (R1 soybean growth stage); ²Plant vigor data were collected at the R3 growth stage (beginning pod); ³Dry aboveground biomass was measured at the R5.1 growth stage (early seed development); ⁴Thousand grain weight; ⁵Efficiency (% increase) reflects treatment gains relative to the untreated control treatment in each level of P fertilization. Treatments 2 and 3 were compared to treatment 1 (which lacks P fertilization), whereas treatments 5 and 6 were compared to the P-fertilized untreated control (treatment 4); ⁶Significantly different means according to Fisher's LSD test ($p \leq 0.05$) are indicated by different lowercase letters within a column.

Table 4. Average values and mean separation results of dependent variables [plant heights, vigor, dry aboveground biomass (DBA), thousand-grain weight (TGW), and yields] gathered from seven maize field trials.

Treatment	Height ¹ (cm)	Vigor ² (1-10 scale)	DBA ³ (g plant ⁻¹)
1. Untreated control - P	179	b ⁶	390
2. EndoFuse (10 mL ha ⁻¹) - P	187	a	417
3. EndoFuse (20 mL ha ⁻¹) - P	187	a	418
4. Untreated control + P	182	b	405
5. EndoFuse (10 mL ha ⁻¹) + P	188	a	395
6. EndoFuse (20 mL ha ⁻¹) + P	189	a	430

Treatment	TGW ⁴ (g)	Maize Yields (kg ha ⁻¹)	Efficiency ⁵ (%)
1. Untreated control - P	268	7396	--
2. EndoFuse (10 mL ha ⁻¹) - P	275	7992	8.0
3. EndoFuse (20 mL ha ⁻¹) - P	272	7910	6.9
4. Untreated control + P	276	7759	--
5. EndoFuse (10 mL ha ⁻¹) + P	278	8120	4.6
6. EndoFuse (20 mL ha ⁻¹) + P	269	8236	6.1

¹Plant height data were collected at early flowering stages (R1 maize growth stage); ²Plant vigor also scored at the R1 growth stage; ³Dry aboveground biomass measured at the R3 growth stage (kernel milk stage); ⁴Thousand grain weight; ⁵Efficiency (% increase) reflects treatment gains relative to the untreated control treatment in each level of P fertilization. Treatments 2 and 3 were compared to treatment 1 (which lacks P fertilization), whereas treatments 5 and 6 were compared to the P-fertilized untreated control (treatment 4); ⁶Significantly different means according to Fisher's LSD test ($p \leq 0.05$) are indicated by different lowercase letters within a column.

plant height in treatments that only received AMF-propagule (seed inoculation) but no P (treatments 2 and 3) matched values recorded at treatments that did receive P fertilization during crop sowing (treatments 5 and 6), as well as being significantly greater than both untreated control treatments (treatments 1 and 4, without and with P) (Table 4). Both

EndoFuse rates displayed similar plant height means, hence indicating that no response to increasing product rates from 10 to 20 ml ha⁻¹ took place for this variable (Table 4). Moreover, plant vigor also experienced a noticeable increase with the addition of AMF propagules, regardless of P fertilization status (Table 4). Mean separation results also indicated that DAB

quantities were less affected by treatments (Table 4). However, the highest EndoFuse rate + P (treatment 6) was found to allow for significantly larger biomass values relative to the untreated control.

Unlike results presented for soybeans, neither seed inoculation with AMF propagules nor P fertilization significantly affected maize's TGW means (Table 4). By contrast, maize yields were significantly increased when seed treatment with EndoFuse was performed, while no significant difference between product rates was observed. Without P fertilization, maize yield increases ranged from 6.9% to 8.0% relative to the untreated control counterpart without P (Table 4). Even when the P fertilization was performed, significant yield increases of 4.6% and 6.1% were recorded at each product rate (10 and 20 mL ha⁻¹), respectively, compared to the P-fertilized untreated control treatment, closely resembling soybean yield results (Table 4). These results agree with Sabia et al. (2015), that reported an 18% gain in dry matter yields, as well as increased P content in the whole plant when maize seeds were inoculated with AMF. Yield increases could be related to many complex processes, such as enhanced ammonium (NH₄⁺) absorption caused by the expression of high- and low-affinity NH₄⁺ transporters such as GintAMT1, GintAMT2, and GintAMT3 in *Rhizophagus irregularis* – one of four AMF species included in EndoFuse – among other benefits (Calabrese et al. 2016). Moreover, it has been reported that AMF-inoculated maize presented greater osmoregulation capacity due to a higher concentration of soluble sugars and electrolytes, which has a direct correlation with maize grain production potential (Feng et al., 2002).

Maize plant vigor and heights, as well as DBA data, were also analyzed separately according to location, given that treatment effects on these variables varied across locations (as indicated by a significant *Treatment x Location* interaction for those variables on Supplementary Table S3). Less noticeable plant height differences were observed across different sites (Fig 2). Itaara/RS was an exception, where a large increase was observed in response to treatments (either AMF or P, Fig 2). A significant increase in plant vigor was observed across all sites (Fig 2). However, the actual intensity (or percent increase) set forth by treatments varied across sites. The largest increases in plant vigor were noticed at Ponta Grossa/PR, Itaara/RS, Iperó/SP, and Planaltina/DF despite contrasting soil P values in these sites (Supplementary Table S1). Furthermore, DBA values were significantly affected by treatments in six out of seven locations, as Iperó/SP showed a different trend. This site was severely affected by heavy rains and wind close to harvest, leading to widespread lodging, which might explain the different responses relative to other experimental sites.

Final considerations

Overall, results obtained for either soybeans or maize indicate that seed treatments containing AMF propagules effectively increase crop yields, among other benefits. Indeed, it has been shown that AMF root colonization in several crops (including maize and soybeans) results in yield increases *c.a.* 37% (McGonigle, 1988). Thereby, AMF seed inoculation could potentially provide a viable alternative to soybeans and maize growers from Brazil, who have broadcast applied P and K right after harvest of the preceding crop. Given the widespread occurrence of 1:1 clay minerals, phosphate can be easily

adsorbed on mineral surfaces in tropical and subtropical conditions (Fink et al., 2014). The association with AMF can mitigate P waste under the extremely high P fertilization rates currently performed in this system. Treating seed with EndoFuse could thus be seen as an important alternative to increase the sustainability of soybean and maize cultivation, especially in a scenario in which yields must be maximized in the coming decades to feed an increasing human population without significant land area expansion.

Material and methods

Field trials, plant material, seed treatment and fertilization

Field trials were conducted in multiple locations during the 2018/2019 growing season in Brazil. A similar protocol consisting of six treatments (Table 1) was conducted across different research sites, in both maize and soybean, as described in Supplementary Table S1.

A novel seed treatment product (EndoFuse®) was applied at two rates (10 or 20 mL ha⁻¹) during seed treatment, either with or without P fertilization (0 or 100%, respectively and accordingly to each location requirements) EndoFuse® contains a combined total of 22,500 AMF propagules mL⁻¹ from four AMF species: *Rhizophagus irregularis*; *Claroideoglossum luteum*; *Claroideoglossum etunicatum*; and *Claroideoglossum claroideum*. Seeds of both maize and soybean were manually treated with EndoFuse® before sowing and seeded with a plot seeder. Treatments were randomly distributed in blocks and consisted of 1: Untreated control without phosphorus fertilization (Untreated control – P); 2: 10 mL ha⁻¹ of EndoFuse applied at seed treatment and without phosphorus fertilization [EndoFuse (10 mL ha⁻¹) – P]; 3: 20 mL ha⁻¹ of EndoFuse applied at seed treatment and without phosphorus fertilization [EndoFuse (20 mL ha⁻¹) – P]; 4: Untreated control with phosphorus fertilization (Untreated control + P); 5: 10 mL ha⁻¹ of EndoFuse applied at seed treatment and with phosphorus fertilization [EndoFuse (10 mL ha⁻¹) + P]; 6: 20 mL ha⁻¹ of EndoFuse applied at seed treatment and with phosphorus fertilization [EndoFuse (20 mL ha⁻¹) + P].

Fertilizers were broadcast applied according to local recommendations. All nutrients were applied equally to all treatments, except for P, which was applied at the rates of 56.4±14.5 (maize trials) and 70.9±9.7 kg ha⁻¹ (soybean trials) of P only in the treatments with P fertilization (100% of P).

Evaluations

Treatments effects on soybeans growth and productivity were evaluated by assessing plant height at early flowering stages (i.e. R1 soybean growth stage), plant vigor at the R3 growth stage (beginning pod), and dry aboveground biomass at the R5.1 growth stage (early seed development) by randomly sampling five plants per plot. Plant vigor was assessed by applying a 1-10 grade scale, at which an untreated control treatment without P fertilization in each block was given a score of 5 and used as a standard for plant vigor comparison purposes. Accordingly, maize plant vigor was assessed 21 days after sowing (DAS), whereas plant heights were assessed at early flowering (R1 growth stage), followed by dry aboveground biomass at R3 (kernel milk stage). Crop yields and thousand-grain weight (TGW) were assessed for both crops by

harvesting 3–8 m² within each plot, and values corrected to 13% humidity as needed.

Experimental design and statistical analysis

Detailed information concerning crop sowing dates, row spacing, and target plant density at each experimental site can be found in Table 2. Experimental units consisted of 12.5–24 m² plots. The number of replications varied from 4 to 6 depending on the location (Table 1). Data were submitted to assumption tests and subjected to analysis of variance (ANOVA) using JAMOVI v 1.2 for Windows (JAMOVI Project, 2020) for unbalanced designs. No data transformation was required. For the ANOVA, Location, Treatment, Block, and Treatment x Location interaction were considered as fixed, independent variables. The results were analyzed across Locations when the interaction between *Treatment x Location* was not significant ($p > 0.05$). Treatments means were compared using Fisher's LSD test when appropriate.

Conclusions

Plant growth-promoting effects are observed in response to the application of AMF propagules contained in EndoFuse in both maize and soybean seeds. Moreover, both doses tested (10 and 20 ml ha⁻¹) were effective to increase the growth traits measured in this work. Interestingly, these positive effects obtained from the AMF inoculation were observed regardless of P fertilization, which is likely related to successful plant root-mycorrhizal fungi associations, resulting from inoculation with arbuscular mycorrhizal fungi propagules contained in the product. Moreover, treatments inoculated with AMF propagule and not fertilized with P presented growth improvements either similar or better than the untreated P-fertilized control treatment, which suggests that AMF inoculation allowed for greater extraction and utilization of native P pools in soils. The yields of both crops were 3.4–8.3% larger when the highest product rate was applied in combination with P fertilization relative to the P-fertilized untreated control, further indicating a greater P use efficiency. Our results show that EndoFuse constitutes a great alternative to facilitate important crops cultivation. These findings can be used as a valuable source of information to change paradigms associated with soybean and maize cultivation in Brazil since P fertilization during sowing is a common practice performed during sowing in this country.

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