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Mycorrhizas obtained from by clay extraction and green manure effect on the growth and nutrition of eucalypts grown in mining area substrate

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SILVICULTURE

ABSTRACT

Background: Clay extraction sites result from mining activities they present sterile, compacted and low-nutrient soils. They have been housing eucalypts crops for wood production. Their management, together with green manure inoculated with symbiont microorganisms, can increase the efficiency of nutrient uptake and reduce the need for chemical fertilization. The aim of the present study is to assess the growth and uptake of macronutrients by *Eucalyptus grandis* seedlings grown in substrate from clay extraction sites, based on intercropping system farming, with green manure inoculated with rhizobia and/or arbuscular mycorrhizal fungi (AMFs). The experiment followed a completely randomized design, with three repetitions, and the following treatments: cultivation of eucalypt (no intercropping) inoculated, or not, with AMFs; eucalypt intercropping system with *Canavalia ensiformis Lam. or Canavalia brasiliensis* Mart ex Benth., inoculated, or not, with AMF´s and/or rhizobia. Isolates of the symbiont microorganism were collected from spontaneous plants grown in clay extraction-site soils. Eucalypt seedlings and green manure were grown, together, in 6 L pots filled with substrate from clay extraction sites. Green manure shoot was cut 45 days after cultivation and eucalypt was harvested 60 days after it.

Results: Inoculation with AMF´s+ rhizobia reduced the C:N ratio and increased N and P acquisition by *C. ensiformis*.

Conclusions: Inoculation of native AMFs from the clay extraction site was effective in boosting the growth and nutrient acquisition of eucalypt plants grown in this substrate, in cultivation intercropped, or not, with *C. ensiformis* or *C. brasiliensis*. It also reduced visible symptoms of nutritional deficiency. Ca, Mg and K concentration in eucalypt plants was not changed by green manure cultivation or by inoculation with AMFs or rhizobia.

Keywords: Arbuscular mycorrhizae; *Canavalia brasiliensis*; *Canavalia ensiformis*; *Eucalyptus grandis*.

HIGHLIGHTS

AMFs isolated from mining area plants stimulate eucalyptus plant growth and nutrition.
Lower C/N ratio of green manure can be obtained through AMF+rhizobia native inoculation.
Green manure and AMF+rhizobia increase nitrogen and phosphorus content in eucalyptus plants.

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INTRODUCTION

The mining industry in Brazil is growing at fast pace, its high profitability rose the interest of Brazilian and foreign investors. However, mineral extraction generates several social and environmental damages, such as biodiversity loss, deforestation, habitat fragmentation, and soil and water quality reduction (Agusdinata et al., 2018). Clay extraction for the manufacture of ceramic products, mainly used in civil construction projects, stands out, negatively, among the mining practices mostly accountable for environmental degradation (Zuquette et al., 2013).

The clay extraction process initially removes the vegetation cover. This procedure is followed by soil fertile-layer removal, but this layer holds organic matter. Therefore, the inocula of symbiotic or non-symbiotic microorganisms are removed from the site and deposited in other areas, due to such a removal (Schiavo et al., 2009). The clay layer is removed from the soil horizon and taken to the ceramic industry, leaving a soil with lower layers with low organic matter and nutrients, without native inocula (Paulucio et al., 2017). These areas are known as clay extraction sites, whose features hinder the recovery process that uses clay extraction sites for agricultural and livestock purposes. Oftentimes these sites are abandoned by their owners due to the high cost to recover soil physical, chemical and biological quality. Yet, eucalypt plantations are often used as firewood supply for the ceramic industry. Although eucalypt species can adapt to sandy soils, with low fertility, poor organic matter and low biodiversity of microorganisms (Jiao et al., 2011), their survival and growth rates in very scarce resource sites are low (Silva et al., 2015).

Implementing eucalypt plantations in consortium with legumes, including tree legumes, has been an alternative for the environmental reclamation of clay extraction sites (Silva et al 2015., Paulucio et al 2017). Fast-growing legumes intended for use as green manure fast cover the soil, increase organic matter rates and recycle nutrients from deeper layers to the topsoil. This process helps the establishment of other plant crops of economic interest, such as eucalypt (Sosa, 2019).

Legumes are capable of fixing atmospheric nitrogen through symbiotic relationships with diazotrophic bacteria - primarily identified by genus *Rhizobium* (Navarro et al, 2021). This process is known as biological nitrogen fixation; it happens when atmospheric nitrogen is fixed on soil and converted into its digestible form for plants. This sequence of factors reduces the need of soil nitrogen fertilization (Arrese-Igor et al; 2021). In addition to diazotrophic bacteria, arbuscular mycorrhizal fungi (AMFs) are symbiont microorganisms found in the soil. They are able to colonize the roots of host plants and to establish mutualistic relationships between the species (Berruti et al., 2016). The formation of extra-radicular mycelium allows hyphae to explore the soil and to share the absorbed water and nutrients with the host; consequently, it promotes plants' growth and development ability (Barea and Kasuya, 2021).

Diazotrophic bacteria and arbuscular mycorrhizae play complementary role in host plants' growth. Interconnection, via hyphae, among mycorrhizal fungi, legumes and associations with rhizobia is a tripartite interaction that can enhance plant nutrition and increase soil sustainability (Ossler et al., 2015; Barea and Kasuya, 2021). This cooperation process between plants and microorganisms plays key role in environmental restoration in ecosystems that have suffered some type of degradation, be it anthropic or natural. It favors community development and the ecosystems ecological succession process (Berruti et al., 2016).

Several studies have pointed out the beneficial effects of nutrient acquisition on eucalyptus, as well as improvements in soil microbial communities when there is consortium of tree legumes in clay extraction sites (Schiavo et al 2009; Paulucio et al, 2017), mainly when plants are inoculated with AMF´s. Although the beneficial effects of the tripartite interaction between legumes, mycorrhizae, and rhizobia are well established, there is little information on the beneficial effects of this interaction on early plant development (Afkhami et al., 2020), especially in the legume intercropping system with other commercial crops. If one takes into consideration the high demand for carbohydrates and nutrients by both plant species and microorganisms in this system; it is possible stating that some of these organisms may acquire more resources at the expense of others. Furthermore, nutrient uptake efficiency may vary depending on the host plant species and on the isolate, itself Suri and Choudhary, (2013); Barea and Kasuya,(2021), as well as on symbiosis' establishment time.

Thus, the hypothesis in the present research advocates that eucalypt plants grown in mining site clay-extraction substrate, intercropped with fast-growth Leguminosae, in association with symbiont microorganisms, have greater growth and substrate-nutrient assimilation ability. The aim of the current study was to assess eucalypt seedlings' growth and macronutrient uptake ability, when they are grown in substrate from clay extraction sites, in consortium with legumes and inoculated with rhizobia and AMFs.

MATERIAL AND METHODS

The experiment was conducted in the greenhouse of Universidade Estadual do Norte Fluminense - Darcy Ribeiro; it followed a completely randomized design, with ten treatments and three repetitions. Treatments were eucalypt cultivation in pure substrate (pure eucalypt); eucalypt grown in substrate inoculated with arbuscular mycorrhizal fungi (AMFs) (eucalypt + AMFs); eucalypt intercropped with legumes, without inoculation with microorganisms; eucalypt intercropped with legumes, inoculated with AMFs; eucalypt intercropped with legumes and inoculated with rhizobia; eucalypt intercropped with legumes and inoculated AMFs and rhizobia. The two legume species used in the experiment were *Canavalia ensiformis* Lam. and *Canavalia brasiliensis* Mart ex Benth, popularly known as 'Brazilian jack bean' and 'wonder bean', respectively.

Arbuscular mycorrhizal fungi were collected from the roots and rhizosphere soil of espontaneous occurrence plants, in a clay extraction site, in Campos dos Goytacazes/RJ region. The site was abandoned for ten years. To verify the presence of mycorrhizae, root samples were placed in glass containers filled with 50% alcohol and kept in refrigerator at 8 ºC, until analysis time. Root segments were discoloured by heating in KOH (5%) at 80 ºC for ten min and washed with deionized water. Samples were acidified in HCl (1%) for 10 min; roots were stained with methyl blue (0.05%) diluted in acid glycerol and heated at 80 ºC until the fragments were evenly colored. Colonization was assessed in 10 root segments (approximately 1 cm long, each) – roots presenting fungal structures such as arbuscules, hyphae, spores, or vesicles were classified as colonized Koske and Gemma, (1989); Grace and Stribley, (1991). The structures of mycorrhizal fungi associated with the roots were analyzed by light microscopy at 200x or 400x magnification (Nikon EclipseE400).

The AMF species of the inoculum were identified through morphological characters by taxonomists of the Biology Department of the Universidade Estadual de Maringá. The inoculum encompassed three species: *Glomus macrocarpum* Tul and Tul; *Acaulospora colombiana* (Spain and Schenck) Kaonongbua, Morton and Bever, (synonymy = *Entrophospora colombiana* Spain and Schenck; Kaonongbua; Morton; Bever) and *Claroideoglomus etunicatum* (Becker and Gerd) Walker and Schüßler (synonymy = *Glomus etunicatum* Becker and Gerd; Walker; Schüßler).

After the mycorrhizal species were identified, a part of the sample collected in the field was registered in the bank of AMF inoculums of the soil Microbiology Sector, at the soil laboratory of Universidade Estadual do Norte Fluminense - Darcy Ribeiro (COFMSOL), under code AMF04. The other part of the sample of roots and rhizospheric soil it was used for AMFs inoculum reproduction for the experiment.

Multiplication was performed in 6-L pots filled with sterilized substrate, autoclaved twice, for 2 h. The substrate comprised soil and sand at 2:1 v/v ratio, and *Braquiaria decumbens* Stapf. seeds (disinfected with 0.5% sodium hypochlorite solution, for 15 min). Brachiaria shoot was cut and discarded four months after inoculation. Roots and substrate remained in the pots for other two months in order to stimulate AMF sporulation. The colonized roots and brachiaria crop soil, with spores, were used as inoculum in the experiment.

Rhizobia nodules of *C. ensiformis* and *C. brasiliensis* were collected from spontaneous plants growing in clay extraction site soils. They were carefully removed from the roots and isolated through streaking on Petri dishes. Subsequently, the isolate was multiplied in YEM-liquid culture medium through orbital shaking (Vincent, 1970). The material multiplied in culture medium was inoculated in seeds of their respective hosts, in cultivation in a sterilized substrate until the beginning of flowering, for rhizobium authentication. The nodules formed were collected, evaluated for their internal pink color and again multiplied in a culture medium. This material was used as inoculant in the plants of the experiment. Nodules' isolation and multiplication followed the procedures adopted by Hungary (1994).

Legume seedlings derived from previously disinfected seeds that were sown in 200 mL plastic cups filled with washed sand (as substrate). Legumes' rhizobia inoculation was performed before sowing by immersing (for one hour) the seeds in YEM culture medium (where the rhizobia was multiplied). Legumes were kept in plastic cups until the time to be transplanted to the pots with eucalypt.

Eucalyptus grandis Wood seeds were previously disinfected through immersion in 70% alcohol, for one minute, and subsequently, in 0.5% sodium hypochlorite solution, for 15 minutes. Then, they were immersed in sterile distilled water for washing purposes. Seeding was performed in 50 mL Styrofoam trays, filled with substrate (mix of washed sand and vermiculite, at 1:2 v/v ratio) and with 15 mL of the inoculum - for the treatments with AMFs.

The substrate in the pots consisted of material taken from a clay extraction site that underwent topsoil replacement. The following chemical features were recorded for the replaced topsoil: pH (water) = 5.6; P = 11 mg kg^{-1}; K = 1.2 mmol kg^{-1}; Ca^{2+} = 16 mmol kg^{-1}; Mg^{2+} = 11 mmol kg^{-1}; Al^{3+} = 1 mmol kg^{-1}; C = 4.8 g kg^{-1} (P and K extraction in Mehlich 1; Ca, Mg and Al extraction in KCl 1 Mol L^{-1}). The growing substrate was added with 20 mg kg^{-1} of P (natural phosphate) before planting; it was homogenized and taken to 6-L pots. The total amount of P applied was based on the recommendation for fertilization in the nursery, for eucalyptus seedlings produced in soil (Barros and Novais, 1999). It was decided to apply 30% of the recommended dose of P was applied so as not to affect mycorrhizal colonization. Another 50 mL of AMF inoculum was added on top of the substrate in treatments with mycorrhizae.

Legume and eucalypt seedlings were transferred to the pots 5 and 20 days after sowing, respectively. They were irrigated with deionized water, on a daily basis, until plant harvesting. Seedlings were treated with two applications of 100 mL pot-1 of nutrient solution (two and thirty days after their transplantation to pots). The solution encompassed the following nutrients (mg L^{-1}): N = 119; P = 15.5; S = 32; K = 110; Ca = 80, Mg = 24, S = 32, Zn = 0.13; Cu = 0.03; Mn = 0.11; Mo = 0.05; B = 0.25. Except for P, the amount of other nutrients applied to the soil was based on the recommendation by Novais et al (1991), with approximately 80% of the recommended value being applied.

Bean plant shoot was cut 45 days after planting, but eucalypt plants remained in the pots with the bean roots. Eucalypt plants' shoot was assessed for height 60 days after legumes were cut; then, they were cut. The roots of the eucalyptus plants were washed on a sieve, under running water, and, later on, in deionized water. Root samples were taken to laboratory and conditioned by immersion in 50% alcohol for further evaluation of mycorrhizal colonization rate. Colonization was assessed in 10 root segments (approximately 1 cm long, each) – roots presenting fungal structures such as arbuscules, hyphae, spores, or vesicles were classified as colonized (Koske; Gemma, 1989; Grace; Stribley, 1991).

The harvested bean and eucalypt plants' shoot was dried in forced air circulation oven, at 65 °C, for 72 h to get the dry matter; subsequently, the material was weighed. The dry material of the three plant species was ground in Wiley-type mill, sieved (in 20 meshes per square inch), and subjected to sulfuric digestion. Phosphorus was determined through colorimetry carried out in spectrophotometer. Samples' total C and N were determined in simultaneous CHNS/O analyzer (Perkin-Elmer, model PE 2400 Series

II). Nutrient content was calculated by multiplying the concentration of each nutrient by shoot dry matter [1], whereas nutrient use efficiency (UE) was calculated based on the equation by Sidique & Glass (1981); where DM is the dry matter (g) and nutrient content was expressed as g plant^{-1}.

UE = {[DM x DM] ÷ content} [1]

Dry biomass and *C. brasiliensis* nutrient content were not determined due to this species' fast growth and tangling in the greenhouse's structure, a fact that made total plant harvest unfeasible. Values recorded for the analyzed variables were subjected to Shapiro-Wilk test, at 5% of significance, to check data normality. Data were subjected to variance analysis and means were tested by Tukey test, at 5% probability level, when values presented normal distribution – procedures were carried out in SAEG software (Statistical and Genetic Analysis System, Euclydes, 1983).

RESULTS

AMFs and rhizobia inoculation in *C. ensiformis* provided dry mass increase, as well as increased concentration and content of available P in the shoot of legume plants in comparison to the control treatment (Table 1). Nitrogen concentration and content also showed higher values in treatments inoculated with microorganisms. No differences were observed in P concentration in species *Canavalia brasiliensis* between treatments based on inoculation with microorganisms and the control (Table 1). Nitrogen concentration treatments based on rhizobia inoculation, with or without AMFs, were higher than that of the control treatment. The C:N ratio was lower in both green manure species due to the inoculation with microorganisms, alone, or in combination to other elements (Table 1).

Mycorrhizal colonization rate in the roots of eucalypt seedlings was significantly higher in treatments based on inoculation with AMFs, with or without, joint inoculation with rhizobia (Figure 1).

Eucalypt shoot dry matter production showed the highest value in the pure culture inoculated with AMF in all treatments and the lowest values for this variable when it was cultivated with *C. ensiformis,* in association with AMF and AMF + rhizobia inoculation (Figure 2A). Dry matter production in each cultivation type has evidenced that inoculation with the microorganisms in eucalypt grown with *C. ensiformis* and *C. brasiliensis* did not cause significant changes in this variable (Figure 2A). The highest plant height values were observed after inoculation with AMFs in the pure crop and when it was intercropped with *C. ensiformis;* the lowest height recorded for this variable was observed under intercrop with *C. brasiliensis* inoculated with AMFs (Figure 2B).

The highest eucalypt shoot N concentration and content was found in plants grown with *C. brasiliensis* inoculated with AMFs in comparison to the other treatments (Figure 3A and Figure 3B), as well as in double inoculation cases, but in this case only for N content (Figure 3B). Inoculation with AMFs did not increase shoot N concentration in eucalypt under pure cultivation (Figure 3A), but it increased the content of this element (Figure 4B). Inoculation did not alter N concentration and content values in eucalypt cultivated with *C. ensiformis* (Figure 3A and Figure 3B). The highest N content was observed in eucalypt cultivation with *C. brasiliensis,* under inoculation with AMFs and AMFs + rhizobia; significantly lower N concentration and content values were observed for the treatment without inoculation (Figure 3A and Figure 3B).

The highest eucalypt shoot P concentration and contents were observed in the pure culture inoculated with the AMFs, and in the culture with *C. ensiformis* and

Table 1: Dry matter, N and P concentration and content, and C:N ratio in *Canavalia ensiformis* Lam. and *Canavalia brasiliensis* Mart ex Benth. shoot due to inoculation with microorganisms (rhizobia and mycorrhizae) grown in pots with *Eucalyptus grandis*.

Analyses	MICROBIOLOGICAL TREATMENT				Coefficient of variation (%)
	Without microorganisms	Rhizobium	AMF	AMF+ Rhizobia	
		Canavalia ensiformis			
Dry matter (g)	22.23 B	24.1 AB	23.4 AB	28.2 A	18
P Concentration (g kg^{-1})	1.77 B	2.23 AB	2.15 AB	2.6 A	24
P Content (mg planta $^{-1}$)	39.4 B	54.1 AB	50.4 AB	74.1 A	32
N Concentration (g kg^{-1})	15.3 B	22.3 AB	24.9 A	21.3 AB	17
N Content (mg planta $^{-1}$)	341.3 B	531.0 AB	586.9 A	599.8 A	27
C/N Ratio	27.1 A	18.4 B	16.8 B	19.6 B	22
		Canavalia brasiliensis			
P Concentration (g kg^{-1})	1.64 A	1.82 A	2.14 A	2.10 A	30
N Concentration (g kg^{-1})	25.2 B	40.1 A	33.6 AB	34.9 A	22
C/N Ratio	16.0 A	9.8 C	12.6 B	12.0 C	15

*Means (3 repetitions) followed by the same uppercase letter did not differ between treatments in the Tukey test, at 5% probability level.

C. brasiliensis, inoculated with AMFs and AMFs + rhizobia (Figure 3C and 3D). The inoculation with microorganisms in eucalypt plants cultivated with *C. ensiformis* or *C. brasiliensis* showed that inoculation with AMFs or AMF + rhizobia increased the P concentration. However, this effect on P content only happened in cultivation with *C. brasiliensis*. The positive effect of inoculation with AMFs on pure cultivation was only observed for P content, since there was no significant increase in the concentration of this element (Figures 3C and 3D). Nitrogen use efficiency (NUE) in eucalypt shoot, under intercrop with *C. brasiliensis,* was higher than that recorded for both *C. ensiformis* and the control (Figure 4A). However, treatments accounted for the lowest phosphorus use efficiency (PUE) values (Figure 4B). The evaluation of each cultivation type pointed towards no differences for NUE and PUE, between treatments; AMFs, with or without rhizobia, significantly reduced the values.

No significant differences were observed for Ca, Mg and K concentration in eucalypt plant shoot in all treatments, regardless of cultivation type or inoculation with AMFs and/or rhizobia (Table 2).

Eucalypt plant shoot, under cultivation with *C. ensiformis* and *C. brasiliensis,* in the control treatment, as well as with inoculation with rhizobia showed chlorosis 45 days after planting - it initially happened in older leaves and extended to younger leaves. Later on, the oldest leaves turned reddish, presented necrotic spots and reduced leaf lamina growth in comparison to the other treatments. Plants inoculated with AMFs and AMFs + rhizobia, whether intercropped (or not) with legumes, showed no symptoms of mineral deficiency.

DISCUSSION

Several research types have highlighted the benefits of consortium crops with legume plants and of inoculation with symbiont microorganisms for eucalypt growth (Schiavo et al., 2009; Paulúcio et al., 2017; Bini et al., 2018). However, no study focused on assessing the potential growth of *Eucalyptus grandis* in crops grown in consortium with *C. ensiformis* or *C. brasiliensis,* and in substrate inoculated with microorganisms from clay extraction sites. Green manure use in eucalypt culture is an important tool to replace organic matter sites that often suffer with the negative impact from topsoil exports.

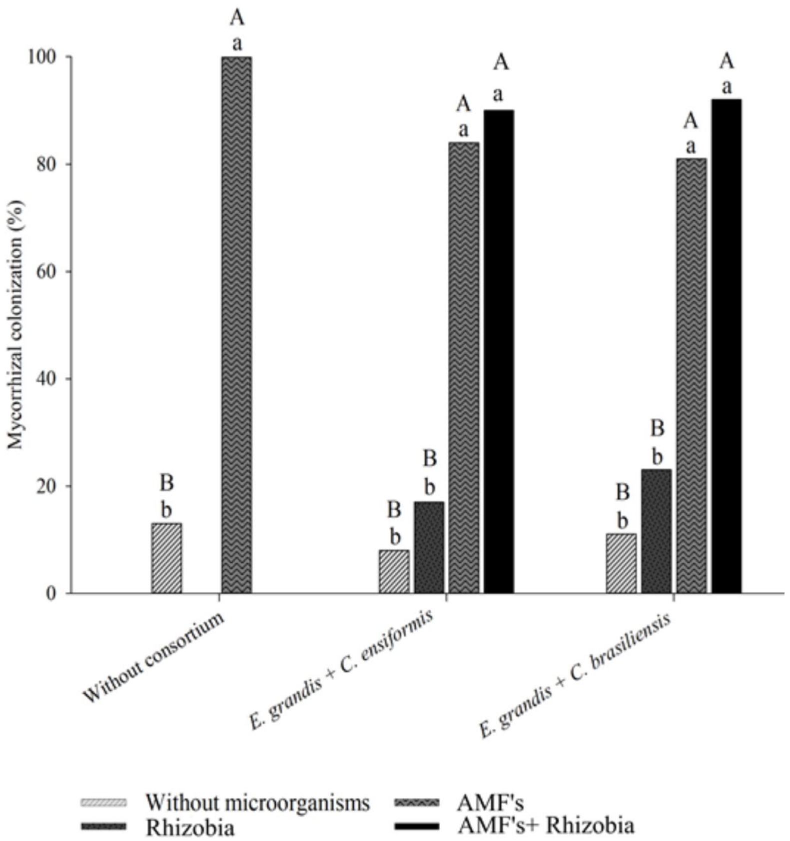

Figure 1: Mycorrhizal colonization of *Eucalyptus grandis* plants as a function of crop type and inoculation with microorganisms. *Means (3 repetitions) followed by the same upper case letter did not differ among the 10 treatments and means followed by the same lower case letter did not differ between microbiological treatments (without microorganisms, rhizobia, AMF’s and AMF’s+ rhizobia) within each crop type (without consortium, in the intercropping system with *E. grandis* and *Canavalia ensiformis* Lam. and with AMF’s *E. grandis* and *Canavalia brasiliensis* Mart ex Benth) in the Tukey test, at 5% probability level. AMF = arbuscular mycorrhizal fungi.

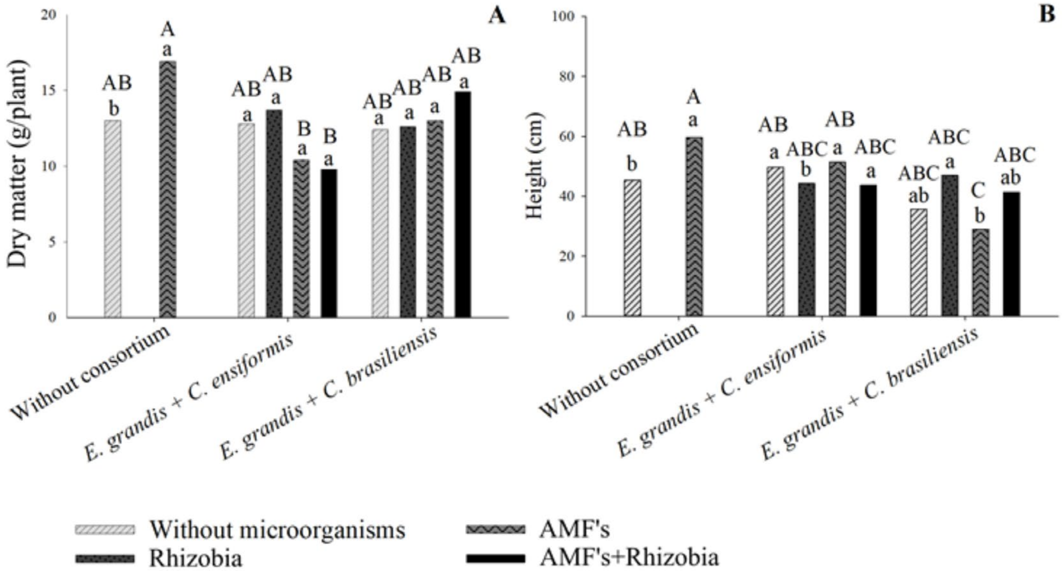

Figure 2: Dry matter production (A) and height (B) of eucalypt plants shoot as a function of crop type and microorganism inoculation. *Means (3 repetitions) followed by the same upper case letter did not differ among the 10 treatments and means followed by the same lower case letter did not differ between microbiological treatments (without microorganisms, rhizobia, AMF's and AMF's+ rhizobia) within each crop type (without consortium, in the intercropping system with *E. grandis* and *Canavalia ensiformis* Lam. and with AMF's *E. grandis* and *Canavalia brasiliensis* Mart ex Benth) in the Tukey test, at 5% probability level. AMF = arbuscular mycorrhizal fungi.

Canavalia ensiformis plants inoculated with AMFs + rhizobia (double inoculation) showed higher dry mass production, lower C:N ratio, and higher N and P uptake than the control (Table 1). The same was observed for *C. brasiliensis,* except for phosphorus uptake. Based on these results, co-inoculation with mycorrhizal fungi and rhizobia in plants induced fast initial shoot growth, besides increasing legume plants' ability to assimilate essential nutrients for their development, even when they are grown in nutrition–deficient soils (Musyoka et al., 2020).

There are situations where the commercial rhizobia inoculant may compete with likely native substrate populations that are naturally more competitive for root establishment, but, in turn, it may be less efficient in biological nitrogen fixation (NBF) (Navarro et al, 2021). Increase in leaf N concentration, due to rhizobia inoculation in *C. ensiformis* and *C. brasiliensis* plants, reached 47 % and 60 %, respectively, in the current study, in comparison to plants that were not inoculated with this symbiont; this finding points out NBF effectiveness; it also led to lower C:N ratio (Figure 4 A and B). This response highlights green manure's higher nutritional and biomass quality when it is inoculated with symbionts.

The high mycorrhizal colonization rate observed in eucalypt plants inoculated with AMFs shows that this inoculation was also effective for plant growth and biomass production. (Figure 1). According to Jiao et al., (2011), some factors can influence colonization, such as the selection of the host species and the inoculation of more than one fungal isolate, which can lead to intraspecific competition and, consequently, reduce colonization. Therefore, colonization by arbuscular mycorrhizal fungi is not always successful. More than 80 % AMFs colonization was observed in all treatments based on inoculum application in the present study. Moreover, no-sterilized substrate contamination with AMFs observed in the treatments without inoculation with AMFs was significantly lower. Campos et al. (2011) observed ages and management differences in commercial *E. grandis* and *Eucalyptus urophylla* ST Blake crops that reached 26% colonization by native arbuscular mycorrhizal fungi, on average, whereas treatments without inoculation ranged from 8% to 23% in the present study.

The highest shoot biomass production was observed for pure eucalypt cultivation inoculated with AMFs (Figure 2). Other studies also showed positive effect of this same native clay extraction site inoculum on *Acacia mangium* Willd (Schiavo et al., 2009; Paulúcio et al., 2017), *Eucalyptus grandis*, *Sesbania virgate* (Cav.) Pers, and *Tectona grandis* Linn. (Rodrigues et al., 2018) cultures in different substrates.

The lowest eucalypt shoot dry biomass, mainly in cultures intercropped with *C. ensiformis,* inoculated with AMFs (Figure 2) may be associated with competition between the plant species for nutrients available in the substrate. Legume plants have fast shoot and root system growth; thus, they may have some advantage in nutrient acquisition and plant development in comparison to eucalypt plants. The 60 days eucalypt plants remained in the pots after legume plant cut were not enough for the acquisition of nutrients deriving from green manure roots' decomposition. Results may change in the field in the long run, and eucalypt plants grown in consortium with leguminous plants, inoculated with microorganisms, may show greater growth than that recorded for pure cultivations. It likely happens because eucalypt root system reaches deeper depths than legume plants, and it reduces the possibility of competition between species. In addition, legume plant shoot in the field will be incorporated by the soil and this process allows more nutrients to enter the soil-plant system.

Adequate N concentration in *E. grandis* plants ranged from 14 to 16 g kg^{-1} (Malavolta et al., 1997). Shoot N concentration did not reach the appropriate range for eucalypt plants in any of the treatments (Figure 3A). Phosphorus concentration ideal range for *E. grandis* seedlings ranges from 1.0 to 2.6 g kg^{-1} (Dell, 1996), and from 1.0 and 1.2 g kg^{-1} (Malavolta et al., 1997). Phosphorus values below the critical level were found in eucalypt plants grown intercropped with *C. ensiformis,* with rhizobia inoculation, and in crops without inoculation with microorganisms in cultures intercropped with *C. ensiformis* and *C. brasiliensis*. Plants showed chlorosis in treatments with deficient N and P concentration; it was initially observed in the oldest leaves and, later, these leaves showed purple coloration - these are typical symptoms of low N availability (Bonila et al., 2021). These symptoms were not observed in treatments based on inoculation with AMFs. This indicates that the AMF inoculated in the plants allowed greater acquisition of P and N (Figure 3) by the eucalyptus, compared to non-inoculation, preventing these plants from showing the visual symptoms of nutritional deficiency.

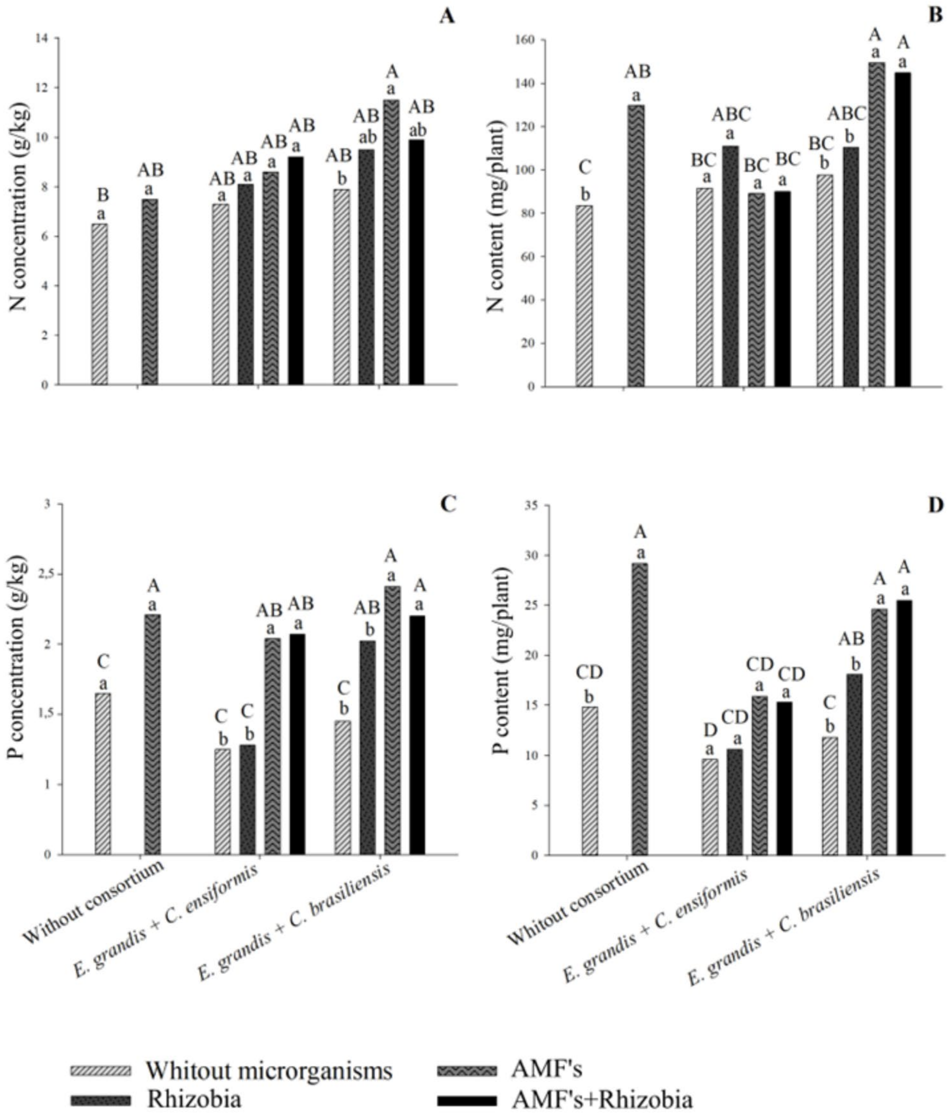

Figure 3: N concentration (A), N content (B), P concentration (C) and P content (D) in the eucalypt plant shoot as a function of the crop type and inoculation with microorganisms. *Means (3 repetitions) followed by the same upper case letter did not differ among the 10 treatments and means followed by the same lower case letter did not differ between microbiological treatments (without microorganisms, rhizobia, AMF’s and AMF’s+ rhizobia) within each crop type (without consortium, in the intercropping system with *E. grandis* and *Canavalia ensiformis* Lam. and with AMF’s *E. grandis* and *Canavalia brasiliensis* Mart ex Benth) in the Tukey test, at 5% probability level. AMF = arbuscular mycorrhizal fungi.

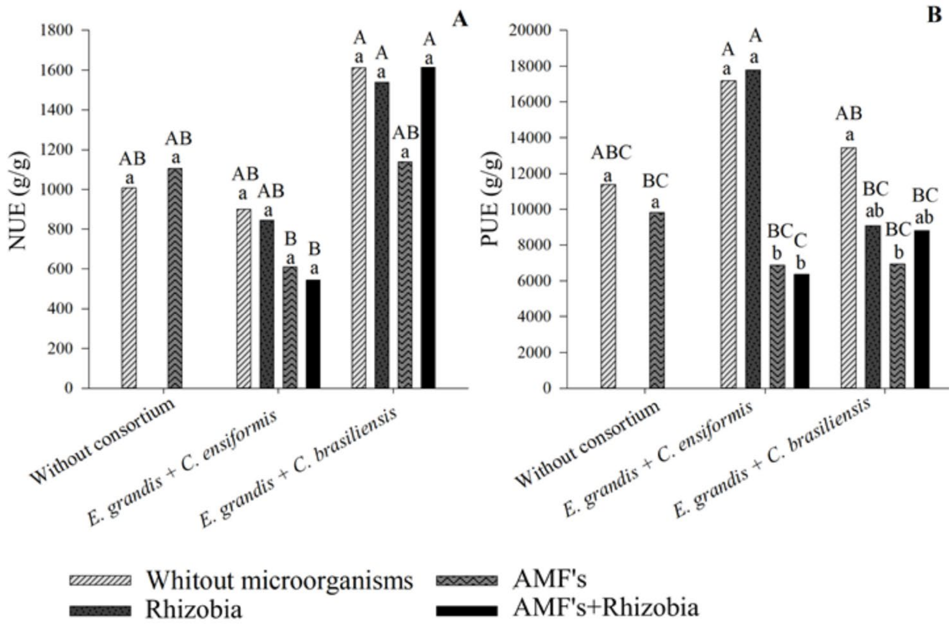

Figure 4: Efficiency of nitrogen use - NUE (A) and phosphorus - PUE (B) in the eucalypt plant shoot as a function of the crop type and inoculation with microorganisms. *Means (3 repetitions) followed by the same upper case letter did not differ among the 10 treatments and means followed by the same lower case letter did not differ between microbiological treatments (without microorganisms, rhizobia, AMF's and AMF's+ rhizobia) within each crop type (without consortium, in the intercropping system with *E. grandis* and *Canavalia ensiformis* Lam. and with AMF's *E. grandis* and *Canavalia brasiliensis* Mart ex Benth) in the Tukey test, at 5% probability level. AMF = arbuscular mycorrhizal fungi.

Table 2: Ca, Mg and K concentration in *Eucalyptus grandis* plants shoot as a function of intercropping with *Canavalia ensiformis* Lam. and *Canavalia brasiliensis* Mart ex Benth. plants and due to inoculation with microorganisms (Rhizobia and Mycorrhizae).

Without consortium		*Canavalia ensiformis*				*Canavalia brasiliensis*				
Without MO	AMF	Without MO	Rhiz	AMF	AMF + Rhiz	Without MO	Rhiz	AMF	AMF + Rhiz	CV (%)
Ca concentration ($g.kg^{-1}$)										
12.1	11.5	12.3	9.3	9.5	9.1	9.8	10.9	11.4	11.5	27
Mg concentration ($g.kg^{-1}$)										
3.0	2.7	3.9	2.8	3.3	2.9	3.2	3.0	3.1	3.0	18
K concentration ($g.kg^{-1}$)										
6.0	6.4	6.7	6.2	6.5	7.0	6.3	5.2	7.2	6.0	25

*The means of the 3 repetitions were compared by F test at 5% probability. Without MO= without microorganisms; AMF = arbuscular mycorrhizal fungi; Rhiz= Rhizobia; CV= Coefficient of Variation.

The highest eucalypt plants' P concentration and content values were observed in treatments based on inoculation with AMFs (Figure 3C and D). This finding confirmed the effectiveness of these symbiont microorganisms in increasing P acquisition by eucalypt, cultivated alone or under consortium with *C. brasiliensis*. Treatments with *C. ensiformis*, inoculated with AMFs and AMFs + rhizobia, showed N concentration increase in eucalypt plants (by 17% and 26%), although it was not significant; P concentration increase reached 105% and 109%, and it may have contributed to absence of mineral deficiency symptoms in these treatments. Treatments presenting the lowest dry matter production responses were observed in crops under intercropping with *C. ensiformis*, inoculated with rhizobia and AMFs + rhizobia – they also presented the lowest NUE.

Decrease in C:N ratio in legume plants (intended for green manure) resulted from inoculation with both AMF and rhizobia; this finding is important because it enables faster legume plants' organic matter decomposition. These plants are mainly grown to benefit the commercial crop, in the present case, eucalypt. Mineralization prevails in organic matter presenting C:N ratio lower than 20:1 (Lucena et al., 2021). Lower C:N ratio values were observed for *C. brasiliensis*, in all treatments; it may have helped in the greater N and P acquisition by eucalypt plants intercropped with this specie. However, legume plant roots remained in the pots to benefit the eucalypt plants. Ca, Mg and K concentrations in eucalypt plants were not reduced by the intercropping system and/or by inoculation with microsymbionts; this finding points out that legume plants did not compete with eucalypt plants for these elements.

Clay extraction site substrates present chemical, physical and biological attribute shortage. Eucalypt cultivation as economic crop and the recovery of these soils from clay extraction sites, through intercropping with legume plants, can be an alternative to increase the efficiency of nutrient uptake by plants, mainly when they are inoculated with symbiont microorganisms. Based on the current results, legume plants inoculated with rhizobia and AMFs showed better nutritional quality, and this is an important green manure feature for the recovery of degraded areas. *E. grandis* cultivation in substrate from clay extraction site grown in consortium with *C. brasiliensis*, and inoculated with AMFs and rhizobia, is an alternative for the establishment of plants in these very environments.

CONCLUSIONS

Inoculation of native AMFs in clay extraction sites is effective for the growth of, and nutrient acquisition by, eucalyptus plants grown in this substrate; moreover, it reduces visible N deficiency symptoms, showed by chlorosis, initially observed in the oldest leaves and, later, these leaves showed purple coloration. Inoculation with AMFs + rhizobia increased *C. ensiformis* and *C. brasiliensis* green manure biomass, and N and P concentration, as well as reduced shoot C:N ratio. Higher N and P concentration and content in eucalypt plants cultivated with *C. brasiliensis* green manure resulted from inoculation with AMF or AMF + rhizobia. Higher P concentration in eucalypt plants intercropped with *Canavalia ensiformis* green manure resulted from inoculation with AMF or AMF + rhizobia, but it did not change N contents or concentration. Ca, Mg and K concentration in eucalypt plants was not changed by green manure cultivation or by inoculation with AMFs or rhizobia.

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REFERENCES

AFKHAMI, M. E.; ALMEIDA, B. K.; HERNANDEZ, D. J.; KIESEWETTER, K. N.; REVILLINI, D. P. Tripartite mutualisms as models for understanding plant–microbial interactions. Current Opinion in Plant Biology, v. 56, p. 28-36, 2020.

AGUSDINATA, D. B.; LIU, W.; EAKIN, H.; ROMERO, H. Socio-environmental impacts of lithium mineral extraction: towards a research agenda. Environmental Research Letters, v. 13, n.12, p. 123001, 2018.

ARRESE-IGOR, C.; HUNGIA, M.; BONILLA, I. Fixação biológica do nitrogênio. In: MARTINEZ, H. E. P.; LUCENA, J. J.; BONILLA, I. Relações Solo-Planta. Bases para a nutrição e produção vegetal. Viçosa (MG), Editora UFV, 2021, 307p.

BAREA, J. M.; KASUYA, M. C. M. Associação Micorrízica e Fertilidade Agrícola. In: MARTINEZ, H. E. P.; LUCENA, J. J.; BONILLA, I. Relações Solo-Planta. Bases para a nutrição e produção vegetal. Viçosa (MG), Editora UFV, 2021, 307p.

BARROS, N. F.; NOVAIS, R. F. Eucalipto. In: RIBEIRO, A. C.; GUIMARÃES, P. T. G.; ALVAREZ, V. V. H. Recomendação para o uso de corretivos e fertilizantes em Minas Gerais - 5ª Aproximação. Viçosa (MG), CFSEMG, 1999, v. 1, p. 303-305

BERRUTI, A.; LUMINI, E.; BALESTRINI, R.; BIANCIOTTO, V. Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. Frontiers in Microbiology, v. 6, p. 155, 2016.

BINI, D.; SANTOS, C. A. D.; SILVA, M. C. P. D.; BONFIM, J. A.; CARDOSO, E. J. B. N. Intercropping *Acacia mangium* stimulates AMF colonization and soil phosphatase activity in *Eucalyptus grandis*. Scientia Agricola, v. 75, p. 102-110, 2018.

BONILLA, I.; ABADIA, J.; BOLANOS, L.; PESTANA, M. Introdução à nutrição vegetal: elementos minerais. In: MARTINEZ, H. E. P.; LUCENA, J. J.; BONILLA, I. Relações Solo-Planta. Bases para a nutrição e produção vegetal. Viçosa, Editora UFV, 2021, 307p.

CAMPOS, D. T. S.; SILVA, M. C. S.; LUZ, J. M. R.; TELESFORA, R. J.; KASUYA, M. C. M. Colonização micorrízica em plantios de eucalipto. Revista Árvore, Viçosa-MG, v.35, n.5, p. 965-974, 2011.

DELL, B. Diagnosis of nutrient deficiencies in eucalypts. In: ATTIWILL, P. M.; ADAMS, M. A. Nutrition of Eucalypts, Australia, Australia, CSIRO, Collingwood, 1996, 440p.

GRACE, C.; STRIBLEY, D. P. A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. Mycological Research, v. 95, p. 1160-1162, 1991.

HUNGRIA, M. Coleta e nódulos e isolamento de rizóbio. In: HUNGRIA, M.; ARAÚJO, R. Manual de métodos empregados em estudo de microbiologia agrícola. Brasília, DF, EMBRAPA, 1994, p.45-59.

JIAO, H.; CHEN, Y.; LIN, X.; LIU, R. Diversity of arbuscular mycorrhizal fungi in greenhouse soils continuously planted to watermelon in North China. Mycorrhiza, v. 21, n. 8, p. 681-688, 2011.

KOSKE, R. E.; GEMMA, J. N. A modified procedure for staining roots to detect VA mycorrhizas. Mycological Research, v. 92, n. 4, p. 486-488, 1989.

MALAVOLTA, E.; VITTI, G. C.; OLIVEIRA, S. A. Avaliação de estado nutricional das plantas. Piracicaba S.P: Associação Brasileira para a pesquisa da Potassa e do Fosfato, 2 ed. 1997, 367p.

MUSYOKA, D. M.; NJERU, E. M.; NYAMWANGE, M. M. E.; MAINGI, J. M. Arbuscular mycorrhizal fungi and Bradyrhizobium co-inoculation enhances nitrogen fixation and growth of green grams (*Vigna radiata* L.) under water stress. Journal of Plant Nutrition, v. 43, n. 7, p. 1036-1047, 2020.

NAVARRO, D. N. R.; SAINZ, J. E. R.; SOUZA, J. L.; SANTOS, R. H. S. Fertilização biológica. In: MARTINEZ, H. E. P.; LUCENA, J. J.; BONILLA, I. Ed. Relações Solo-Planta. Bases para a nutrição e produção vegetal. Viçosa, Editora UFV, 2021, 307p.

NOVAIS, R. F.; NEVES, J. N. L.; BARROS, N. F. Ensaio em ambiente controlado. In: OLIVEIRA, A. J.; GARRIDO, W. S.; ARAÚJO, J. D.; LOUREÇO, S. Métodos de Pesquisa em Fertilidade do Solo. Brasília (DF), EMBRAPA, 1991, p189-249.

OSSLER, J. N.; ZIELINSKI, C. A.; HEATH, K. D. Tripartite mutualism: facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. American Journal of Botany, v. 102, n. 8, p. 1332-1341, 2015.

PAULUCIO, V. D. O.; SILVA, C. F. D.; MARTINS, M. A.; PEREIRA, M. G.; SCHIAVO, J. A.; RODRIGUES, L. A. Reforestation of a Degraded Area with Eucalyptus and Sesbania: Microbial Activity and Chemical Soil Properties. Revista Brasileira de Ciência do Solo, v. 41, p. 1-14, 2017.

RODRIGUES, L. A.; BARROSO, D. G.; FIGUEIREDO, F. A.; ASSIS, A. M. M. Fungos micorrízicos arbusculares no crescimento e na nutrição mineral de mudas de *Tectona grandis* L. F. Ciência Florestal, v. 28, p. 25-34, 2018.

SCHIAVO, J. A.; MARTINS, M. A.; RODRIGUES, L. A. Avaliação nutricional de mudas de *Acacia mangium*, *Sesbania virgata* e *Eucalyptus camaldulensis* inoculadas com fungos micorrízicos, em casa-de-vegetação e em cava de extração de argila. Acta Scientiarum. Agronomy, v. 31, n. 4, p. 701-707, 2009.

SILVA, C. F. D.; CARMO, E. R. D.; MARTINS, M. A.; FREITAS, M. S. M. D.; PEREIRA, M. G.; SILVA, E. M. R. D. Deposition and nutritional quality of the litter of pure stands of *Eucalyptus camaldulensis* and *Acacia mangium*. Bioscience Journal, v. 31, n. 4, p. 1081-1091, 2015.

SURI, V. K.; CHOUDHARY, A. K. Effect of Vesicular Arbuscular-Mycorrhizal Fungi and Phosphorus Application through Soil-Test Crop Response Precision Model on Crop Productivity, Nutrient Dynamics, and Soil Fertility in Soybean-Wheat-Soybean Crop Sequence in an Acidic Alfisol. Communications in Soil Science and Plant Analysis, v. 44, n. 13, p. 2032-2041, 2013.

ZUQUETTE, L. V.; RODRIGUES, V. G. S.; PEJON, O. J. Recuperação de áreas degradadas. Revista Engenharia Ambiental: Conceitos, Tecnologia e Gestão, v. 13, p. 589-619, 2013.