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The phytoprotective effects of arbuscular mycorrhizal fungi on *Enterolobium contorstisiliquum* (Vell.) Morong in soil containing coal-mine tailings

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ABSTRACT
The purpose of this study was to evaluate the effects of arbuscular mycorrhizal fungi (AMF) on pacara earpod tree (*Enterolobium contorstisiliquum*) growth and phytoprotection in soil containing coal-mining waste. A greenhouse experiment was carried out with three inoculation treatment groups (non-inoculated, inoculated with *Rhizophagus clarus*, and inoculated with *Acaulospora colombiana*) in two substrates (0 or 30% tailings). After 90 days the seedlings were collected to quantify growth parameters, quality, mycorrhizal root colonization rate, and leaf content of chlorophylls and carotenoids. Macronutrients were quantified in the shoots; Cu, Zn, and Mn levels were measured in the shoots and roots; and glomalin content was measured in the rhizosphere. Colonization by *A. colombiana* (40%) promoted phytoprotection and better growth in seedlings planted in partial tailing substrate, due to the lower Cu (1.04 mg kg^{-1}) and Zn (13.4 mg kg^{-1}) levels in shoot dry mass and reduced translocation of these elements to the shoots. *A. colombiana* increased soil glomalin concentrations (2.98 mg kg^{-1}) and the accumulation of nutrients necessary for synthesizing chlorophylls and carotenoids in the leaves. Colonization by *R. clarus* (81%) produced no phytoprotective effects.

KEYWORDS
Plant protection; AMFs; heavy metals; pacara earpod tree; degraded areas

Introduction

Approximately 30% of global energy is derived from coal (British Petroleum 2016). One negative impact that coal mining has on the environment is the removal of vegetation and topsoil, where organic matter and the seed bank are concentrated. Large amounts of mine tailings have been disposed off on the Brazilian soil, which has had severe environmental impacts due to the high impurity levels of local coal (Amaral filho *et al.* 2013). Although the technical criteria for recovering such areas recommend removing waste from the site, some residues may remain in the soil (Ação Civil Pública 2016). This further undermines potential environmental resilience and efforts to rehabilitate, recover, or restore coal-mining sites, due to the resulting low organic matter content, available nutrients and pH, as well as possible contamination by heavy metals (Silva *et al.* 2013). This poses risks to humans and biodiversity, causing severe environmental and social problems in coal-mining areas, as has been reported in the state of Santa Catarina, Brazil (Silva *et al.* 2013).

Phytoremediation, a technique used to help re-vegetate areas degraded by mining, involves two main processes: phytostabilization and phytoextraction (Ali *et al.* 2013). Those low-cost processes minimize metal bioavailability in the soil (Wan *et al.* 2016). The efficiency of this technique may be increased by using native plant species due to their better growth, adaptation to the local climate, resistance to pathogens, increased biomass production, and facilitation of ecological processes (Shabani and Sayadi 2012).

The pacara earpod tree [*E. contorstisiliquum* (Vell.) Morong], Leguminosae, is a species native to Brazil and can be found in different regions of Latin America. Previously research has been conducted to assess its development potential in soils contaminated by heavy metals. This tree is a promising species for phytoremediation and the recovery of degraded areas because it has proven to be more tolerant than other native leguminous species such as the Brazilian orchid tree (*Bauhinia forficata*) to soil contaminated by Cu (Silva *et al.* 2015).

In order to survive in heavily impacted areas, such as those containing coal-mine tailings, plants require association with soil microorganisms, such as arbuscular mycorrhizal fungi (AMF) (Smith and Read 2008). One study on gold-mining areas contaminated with As reported that *Acaulospora* was the most abundant AMF genus (Schneider *et al.* 2013), while another on the molecular diversity of AMF in a mining area contaminated with Mn found that the genera *Glomus* and *Rhizophagus* were the most common, being more tolerant to metal contamination (Wei *et al.* 2014).

Arbuscular mycorrhizae are the most important type of mycorrhiza for phytoremediation (Cabral *et al.* 2015), since they increase soil mineral uptake, including heavy metals (Soares *et al.* 2001). On the other hand, mycorrhizas may contribute to phytoprotection by enhancing nutritional and physiological processes in plants (Smith and Read 2008; Doubková and Sudová 2016; Ferreira *et al.* 2015). Such processes include metal immobilization in roots (Soares *et al.* 2001) and fungal mycelium (Cabral et a. 2015), which may result in reduced

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levels of diffusion to plant shoots (Cabral et a. 2015; Ferreira *et al.* 2015). Another phytoprotective effect mediated by AMF is the complexation of chemical elements with glomalin molecules, which are exuded in the soil by these fungi, possibly leading to lower bioavailability of heavy metals (Vodnik *et al.* 2008; Wu *et al.* 2014) and increased plant growth.

The performance level of this mycorrhizal association depends on the plants and fungi involved, as well as the environmental conditions and coevolution time of the species involved (Schweiger *et al.* 2014). However, few studies have been carried out to verify the potential of using AMF with native tree species for soil decontamination and recovery in degraded coal-mining areas.

The purpose of this study was to evaluate the effects of two AMF isolates, associated or not with coal-mine tailings, on the growth, metal phytoextraction potential, and phytoprotective capacity of pacara earpod tree [*E. contorstisiliquum* (Vell.) Morong] seedlings.

Material and methods

Experimental design

The experiment was conducted for 90 days in a greenhouse in southern Brazil (27°35′54.21″ S and 48°30′56.40″ W) using 300-cm^3 polypropylene pots. The treatments were arranged in a (3 × 2) factorial design of three inoculation groups (non-inoculated, inoculated with *R. clarus* and inoculated with *A. colombiana*) and two waste levels (without tailings "−T" and with tailings "+T"), with 12 completely randomized replicates. The waste dosage had been previously determined in a 90-day greenhouse experiment (non-autoclaved soil with 0%, 15%, 30%, and 45% tailings added) where 30% and 45% doses reduced seedling shoot dry mass (SDM) (data not presented).

Soil and tailing collection and soil characterization

Soil and tailings were collected from the 0–20-cm layers in areas surrounding a coal-mining site near Criciúma, Santa Catarina (28°35′16.21″ S and 49°26′48.43″ W). Samples were sieved (4.0 mm mesh) and fertilized with 150 mg of P per kg (as triple superphosphate). The substrates were put into pots and autoclaved twice. After 30 days, three samples were collected from each substrate for physical and chemical characterization, according to Tedesco *et al.* (1995). Soon afterward, the soil texture was assessed by the pipette method (EMBRAPA 1997). Potentially available forms of Cu, Zn, Mn, P, and K were quantified after extraction with Mehlich-1 solution. Total organic carbon was determined by the Walkley-Black method and total N by sulfuric digestion at 360°C. Nitro-perchloric digestion (nitric acid and perchloric acid 6:1, v:v) at 180°C was used for total extraction of Cu, Zn, and Mn. Cu and Zn levels were above the legal tolerance levels (CETESB 2016), which are 35 and 60 mg kg^{-1}, respectively.

Mycorrhizal inocula and seeds

Rhizophagus clarus and *A. colombiana* inocula were supplied by the International Glomeromycota Culture Collection (www.furb.br/cicg/index.php?lang=EN). The AMF were multiplied with *Brachiaria decumbens* as a host plant in an autoclaved mixture of soil, sand, and commercial substrate (1:3:1; v:v:v). Spore counts were 8.0 spores/ml for *R. clarus* and 3.0 spores/ml for *A. colombiana*.

Since environmental regulations require native species for re-vegetation of degraded areas, pacara earpod tree [*E. contorstisiliquum* (Vell.) Morong] seeds were acquired from Rio Grande do Sul Agricultural Research Foundation (www.fepagro.rs.gov.br), which regularly supplies companies and institutions in charge of land restoration. Seed dormancy was broken by immersion in sulfuric acid (98%) for 15 minutes and rinsing in distilled water (Fowler and Bianchetti 2000).

Sowing and growing conditions

At sowing, the AMF inoculum was added (2.0 cm depth) in amounts resulting in approximately 200 spores per pot (Ferreira *et al.* 2015). Non-inoculated controls received 50 ml of a suspension of each inoculum that had been filtered through a paper filter. Three seeds were planted in each pot, but the sprouts were thinned to one per pot. Each pot received 1.0 ml of *Bradyrhizobium elkanii* (SEMIA 6159 - BR 4406) inoculum multiplied in liquid yeast-extract culture medium (Vincent 1970).

A nutritive solution without P or N was added weekly (20 ml per pot) (Hoagland and Arnon 1950). The soil was kept at about 70% of field capacity by adding distilled water. The mean air temperature in the greenhouse was 27.7°C throughout the experiment.

Quantified variables

The SPAD index (soil plant analysis development) was obtained by testing three leaves in the middle section of each seedling with a digital chlorophyll meter (Minolta SPAD model 502). In order to quantify chlorophylls (a, b, total) and carotenoids, 100-mg samples of leaves from the middle section of plants were collected and incubated for 2 hours at 65°C with 7.0 ml of dimethyl sulfoxide. Total volume was adjusted to 10 ml, and absorbance was measured by spectrophotometry (Pro-analysis, V1600 Model) (Hiscox and Israelstam 1979). Chlorophylls were quantified using Wellburn's equations (Wellburn 1994).

After 90 days, seedling height (H) and stem diameter (SD) were measured and the shoots were separated from the roots. The roots were washed sequentially with tap water, ethylenediamine tetracetic acid solution (0.02 mol L^{-1}), and distilled water. Root mycorrhizal colonization was measured under a light microscope (McGonigle *et al.* 1990) after clearing and staining (Koske and Gemma 1989). The root nodules were collected, counted, dried, and weighed. The plant shoots and roots were dried and weighed. SDM, root dry mass (RDM), and total dry mass (TDM) were used to calculate the Dickson quality index value (DSQ) (Dickson *et al.* 1960), as follows:

$$\begin{aligned}\mathrm{DSQ} = \mathrm{TDM}\ (\mathrm{g}) / [(\mathrm{H}\ (\mathrm{cm}) / \mathrm{SD}\ (\mathrm{mm})) \\ + (\mathrm{SDM}\ (\mathrm{g}) / \mathrm{RDM}\ (\mathrm{g}))]\end{aligned}$$

The dried plant material underwent either nitro-perchloric digestion (as previously described) to determine total Cu, Zn,

Table 1. Granulometric fractions and chemical attributes of soil without (-tailings) and with coal-mine tailing addition (+tailings), 30 days after phosphate fertilization and autoclave sterilization.

Attribute	Unit	- tailings		+ tailings	
Sand	g kg^{-1}	268	±1.1$^{(1)}$	354	±4.2
Silt	g kg^{-1}	454	±2.9	389	±7.2
Clay	g kg^{-1}	278	±1.5	257	±3.0
pH$_{H2O}$	–	4.4	±0.05	4.1	±0.04
Aluminum	cmol$_c$ kg^{-1}	6.2	±0.1	5.6	±0.3
TOC	g kg^{-1}	19.6	±1.1	25.8	±0.13
Nitrogen$_{total}$	g kg^{-1}	0.5	±0.03	0.4	±0.01
Phosphorus$_{resin}$	mg kg^{-1}	77	±3.7	82	±5.3
Phosphorus$_{extractable}$	mg kg^{-1}	132	±9.9	134	±3.61
Potassium$_{extractable}$	mg kg^{-1}	60	±2.1	44	±0.4
Calcium$_{extractable}$	cmol$_c$ kg^{-1}	2.7	±0.1	2.6	±0.17
Magnesium$_{extractable}$	cmol$_c$ kg^{-1}	0.5	±0.1	0.7	±0.12
Copper$_{extractable}$	mg kg^{-1}	7.1	±0.3	11.1	±0.36
Manganese$_{extractable}$	mg kg^{-1}	58	±1.1	64	±2.2
Zinc$_{extractable}$	mg kg^{-1}	34	±2.3	42.1	±2.49
Copper$_{total}$	mg kg^{-1}	50	±7.1	51	±3.2
Manganese$_{total}$	mg kg^{-1}	411	±8.5	419	±9.2
Zinc$_{total}$	mg kg^{-1}	256	±9.2	242	±9.3

TOC, total organic carbon.
$^{(1)}$Mean standard deviation, n = 3.

and Mn contents in the shoots and roots, or to to determine total N by sulfuric digestion (as previously described) to quantify total macronutrients in the shoots (EMBRAPA 1997). Levels of Ca, Mg, Cu, Zn, and Mn were measured by atomic absorption spectrophotometery, P by spectrophotometery, K by flame photometery, and N by the micro-Kjeldahl method.

Total glomalin-related protein in the soil was measured with a Bradford assay (Bradford 1976), modified according to Wright and Upadhyaya (1998). Soon afterward, 1.0 g of rhizospheric soil (soil in contact with roots) was removed with a brush and placed in a falcon tube (15 ml) with 8 ml of sodium citrate (50 μmol), which was then autoclaved for 1 hour at 121°C, with subsequent centrifugation at 1,888 × g. The process was repeated until the supernatant was light yellow (Wu *et al.* 2014).

Statistical analysis

The data were subjected to analysis of variance (ANOVA). When significant differences were detected, the means were separated with the Tukey test ($p < 0.05$) in SISVAR software

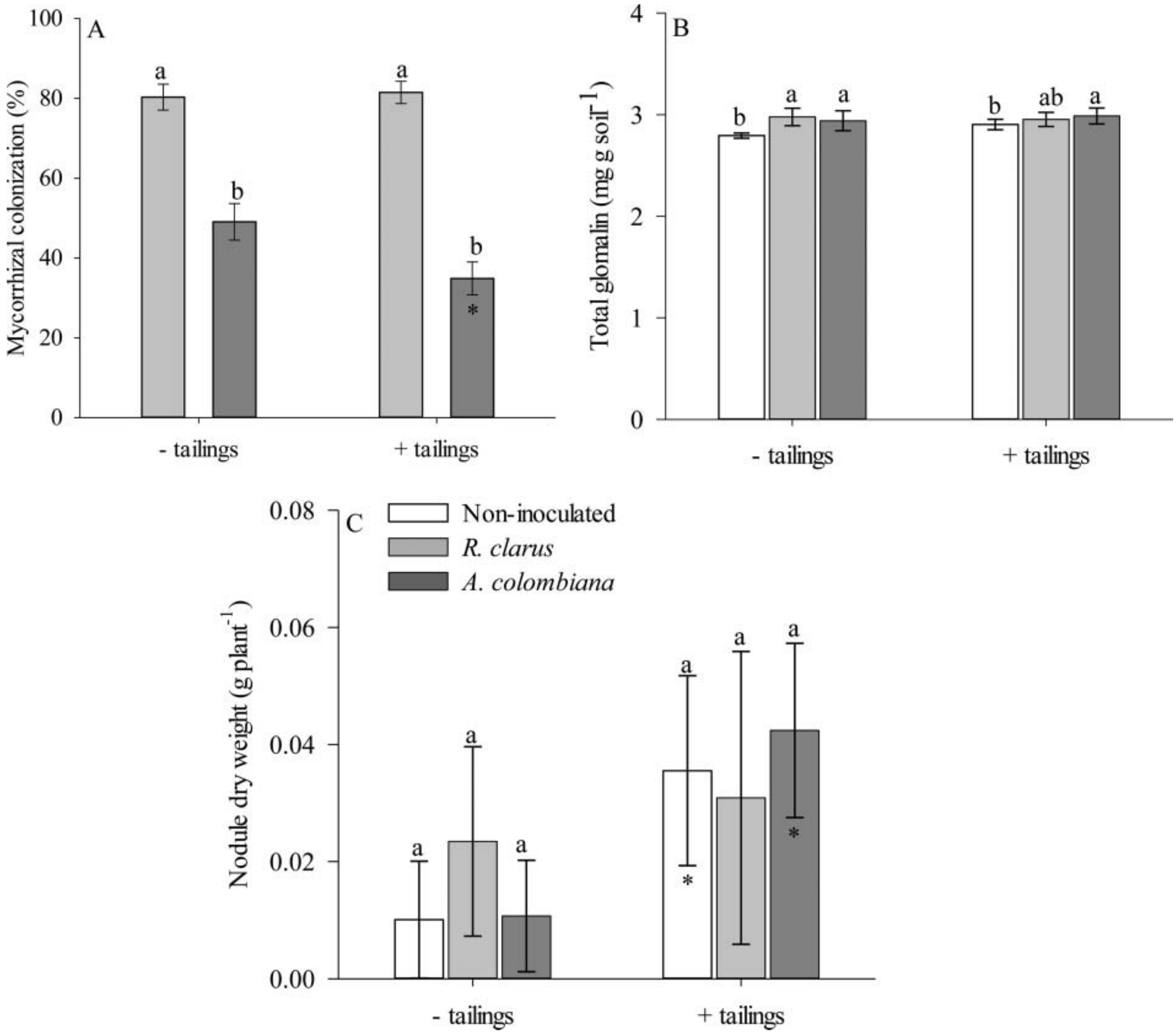

Figure 1. Mycorrhizal colonization (A), total glomalin (B), and nodule dry weight (C) of pacara earpod tree seedlings, in a greenhouse, in soil with or without coal-mine tailing and non-inoculated or inoculated with either *Rhizophagus clarus* or *Acaulospora colombiana*. ^{1}Means followed by the same letter are not different in the effect of AMF inoculation within each level of tailings. *Significative effect of tailings within each AMF inoculation treatment (Tukey test, $p < 0.05$, n = 12).

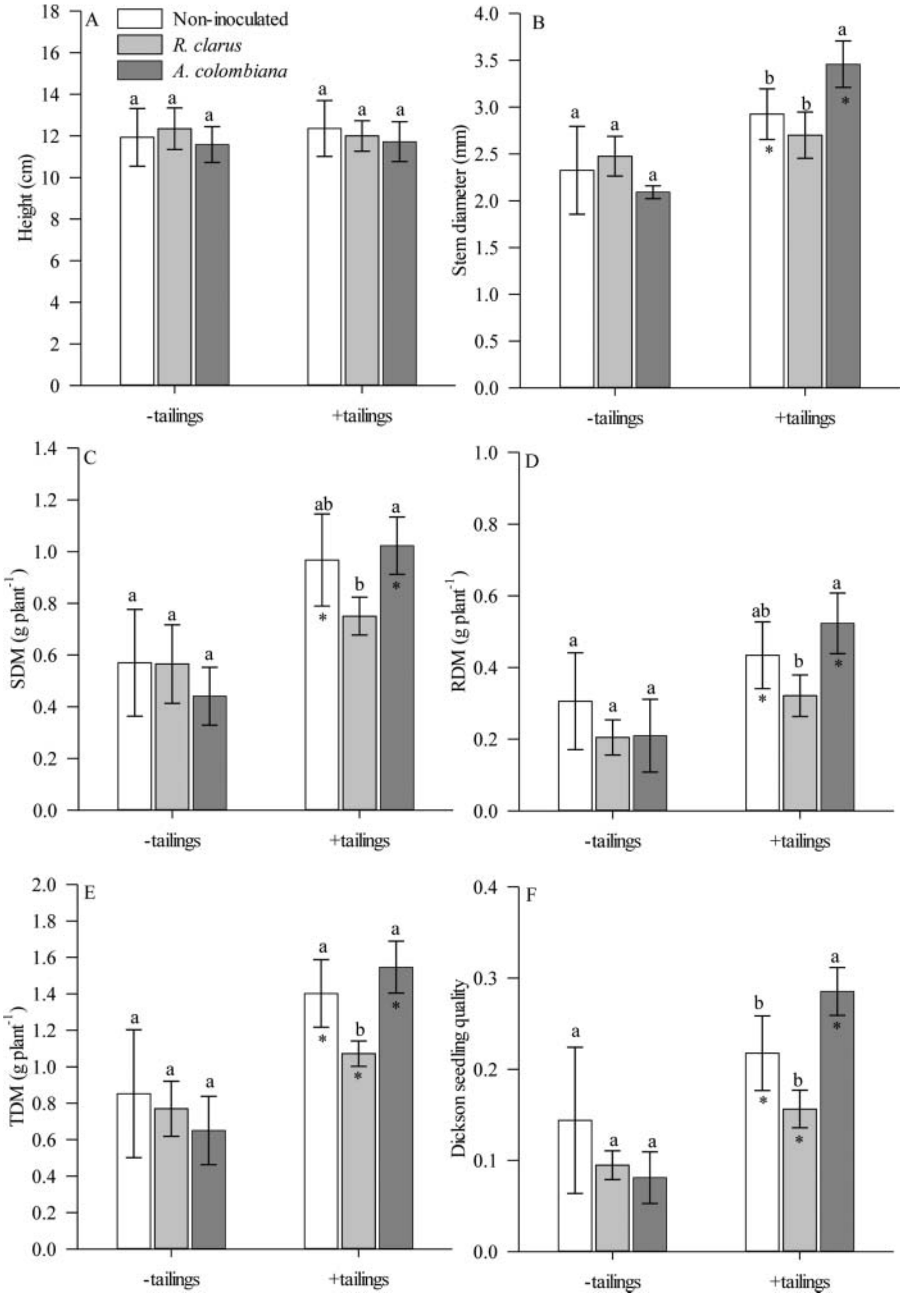

Figure 2. Height (A), stem diameter (B), shoot dry matter (SDM) (C), root dry matter (RDM) (D), total dry matter (TDM) (E), and Dickson seedlings quality (F) of pacara earpod tree plants grown in a greenhouse, in soil with or without coal-mine tailing and non-inoculated or inoculated with either *Rhizophagus clarus* or *Acaulospora colombiana*. [1]Means followed by the same letter are not different in the effect of AMF inoculation within each level of tailings. *Significative effect of tailings within each AMF inoculation treatment (Tukey test, $p < 0.05$, $n = 12$).

(Ferreira 1998). Graphics were generated with SigmaPlot, version 12.5. When necessary, the results were transformed to meet ANOVA assumptions. Four replicates of each dependent variable were used for principal component analysis (PCA) in CANOCO, version 4.5 (Ter Braak and Smilauer 1998).

Results and discussion

Soil characteristics

The addition of tailings led to increased sand content and changes in several chemical attributes (Table 1). The partial tailing substrate had lower pH and exchangeable K, as well as higher organic matter and higher Cu, Mn, and Zn content.

Mycorrhizal colonization, glomalin content, and nodulation

Seedlings inoculated with *R. clarus* had a twofold higher root colonization index than those inoculated with *A. colombiana* (Figure 1a). *R. clarus* has previously shown high potential for colonizing *Crotalaria juncea* grown in Cu-contaminated soil (Ferreira *et al.* 2015). Pacara earpod trees are conducive to AMF colonization, since plants inoculated with *Gigaspora margarita* and *Glomus*

Table 2. SPAD index, chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl Total), and carotenoids (Carot.) in pacara earpod tree seedlings grown in a greenhouse, in soil with or without coal-mine tailing, and non-inoculated or inoculated with *Rhizophagus clarus* or *Acaulospora colombiana*.

	Ni-T		*Rc*-T		*Ac*-T		Ni + T		*Rc* + T		*Ac* + T	
SPAD Cv = 18.75%	24.1	b[1]	30.9	a	28.1	a	24.9	b	32.7	a	32.3	a
							μg mL					
Chl a Cv = 22.45%	13.5	a	15.8	a	14.9	a	18.6	a*	17.1	a	16.6	a
Chl b Cv = 29.08%	3.66	b	5.14	a	3.98	a	5.18	a*	5.16	a	4.86	a
Chl Total Cv = 22.45%	17.2	a	21.0	a	18.9	a	23.8	a*	22.3	a	21.4	a
Carot. Cv = 17.11%	3.21	a	3.60	a	3.59	a	3.96	a*	3.67	a	3.46	a

Ni, non-inoculated; *Rc, Rhizophagus clarus*; *Ac, Acaulospora colombiana*; -T = without tailing; +T = with tailing; Cv = coefficient of variation.

[1] Means followed by the same letter in each same line are not different within level of tailings. *indicates significant effect of tailing within each level of inoculation (Tukey test, $p < 0.05$, $n = 12$).

etunicatum had 84% of their root volume colonized (Moreira *et al.* 2010). Colonization by *A. colombiana* reached approximately 40% (Figure 1a), which indicates that this AMF has a lower affinity with pacara earpod trees. Tailings had no effect on *R. clarus* mycorrhizal colonization (Figure 1a), but that of *A. colombiana* was lower when tailings were added to the substrate (Figure 1a). It could be that *A. colombiana* produced better plant growth conditions in partial tailing substrate, which would have reduced colonization, since plants with adequate growth conditions do not depend on mycorrhizal colonization to alleviate negative environmental effects (Siqueira *et al.* 1999).

The glomalin content was higher in substrates with *A. colombiana*-inoculated seedlings than in those inoculated with *R. clarus*, irrespective of tailing (Figure 1b). The glomalin-related protein found in treatments without mycorrhizal inoculation may have originated from native AMFs already present in soil from the mining area (Schneider *et al.* 2013; Wei *et al.* 2014). Janos *et al.* (2008) suggest that Wright and Upadhyaya's glomalin quantification method (Wright and Upadhyaya 1998), which was used in our study, may extract a "pool" of similar proteins, inferring that not every protein extracted is truly glomalin. However, the high levels of glomalin-related protein found in AMF-inoculated substrates suggest that 3 months were enough to increase the content of that protein in substrate containing coal-mine tailings (Figure 1b). Total glomalin is more stable in the environment than its easily extractable fraction, hence its importance in studies on phytoremediation and contaminated soil recovery (Vodnik *et al.* 2008; Wu *et al.* 2014). The fact that protein can adsorb 1.6–4.3 mg of Cu (González-Chávez *et al.* 2004), 3.3–4.8 mg of Zn (Cornejo *et al.* 2008), 2.23–4.42 mg of Mn (Chern *et al.* 20007) per gram favors initial plant growth in metal-contaminated soil, as demonstrated in *A. colombiana*-inoculated seedlings planted in 30% tailing substrate (Figure 2b–d and F).

The addition of tailings increased nodule weight in controls and *A. colombiana*-inoculated seedlings (Figure 1c), which may have contributed to the improved growth and seedling quality (Figure 2). In general, pacara earpod trees have shown an association with diazotrophic bacteria in low-fertility soils, resulting in good seedling quality (Moreira *et al.* 2010).

Plant growth and seedling quality

Seedling height was affected by neither the addition of tailings nor mycorrhizal inoculation (Figure 2a). However, seedling SDM and RDM were higher in *A. colombiana*-inoculated and control seedlings grown in partial tailing substrate (24% and 38%, respectively), than in those treated with *R. clarus* (Figure 2c and d). There was no difference in TDM between *A. colombiana*-inoculated seedlings and controls planted in partial tailing substrate (Figure 2e), although those treated with *A. colombiana* had greater stem diameter (Figure 2b). These results affected seedling quality, since the highest Dickson quality index was found in *A. colombiana*-inoculated seedlings planted in partial tailing substrate, which was 45% and 24% higher than *R. clarus*-inoculated seedlings and controls, respectively ($p < 0.05$) (Figure 2f). The addition of tailings improved this index in both inoculation treatment types, suggesting that the chemical changes and increased sand content in the partial tailing substrate may have improved the physical conditions for plant growth.

The plant growth and seedling quality data suggest that there were synergistic effects in the tripartite interaction of *A. colombiana, B. elkanii*, and plants in soil containing 30% mine tailings. A positive effect of this tripartite interaction was also found in plants inoculated with a mixture of AMF genera in low-fertility soil (Moreira *et al.* 2010). Antunes *et al.* (2006) suggested that the improved performance of plants associated with AMF is due to increases in specific rhizosphere flavonoids, especially in disturbed soils, such as those in mining areas. In addition to their antioxidant function, flavonoids such as daidzein and coumestrol can stimulate AMF hyphal growth and root colonization (Xie *et al.* 1995). Furthermore, genistein acts as an auxin transport inhibitor, which facilitates nodulation (Haichar *et al.* 2014). Due to its potential to mitigate the effects of stress and increase plant survival, a successful tripartite interaction must be considered in the development of re-vegetation programs in degraded areas (Smith and Read 2008).

SPAD index, chlorophylls, and carotenoids

Mycorrhizal inoculation increased SPAD index values without affecting chlorophyll or carotenoid levels, except for chlorophyll b in normal soil (Table 2). In addition to the benefits of associating with *Acaulospora* and *Bradyrhizobium*, the addition of tailing increased chlorophyll and carotenoid levels (Table 2), whose molecules are associated with antioxidant mechanisms in plants subjected to stress conditions (Ahmed *et al.* 2010). Carotenoids are related to phytoprotection mechanisms and regulate photosynthetic mechanisms (Giuliano 2014), contributing to seedling development in contaminated soil. These results indicate the potential contribution that mycorrhizal inoculation of native tree species may make toward re-vegetating soils contaminated with coal-mining waste.

Cu, Zn, and Mn contents in shoots and roots

The highest shoot Cu content was found in *R. clarus*-inoculated seedlings in normal soil, while the lowest Cu content was found in *A. colombiana*-inoculated seedlings in partial tailing

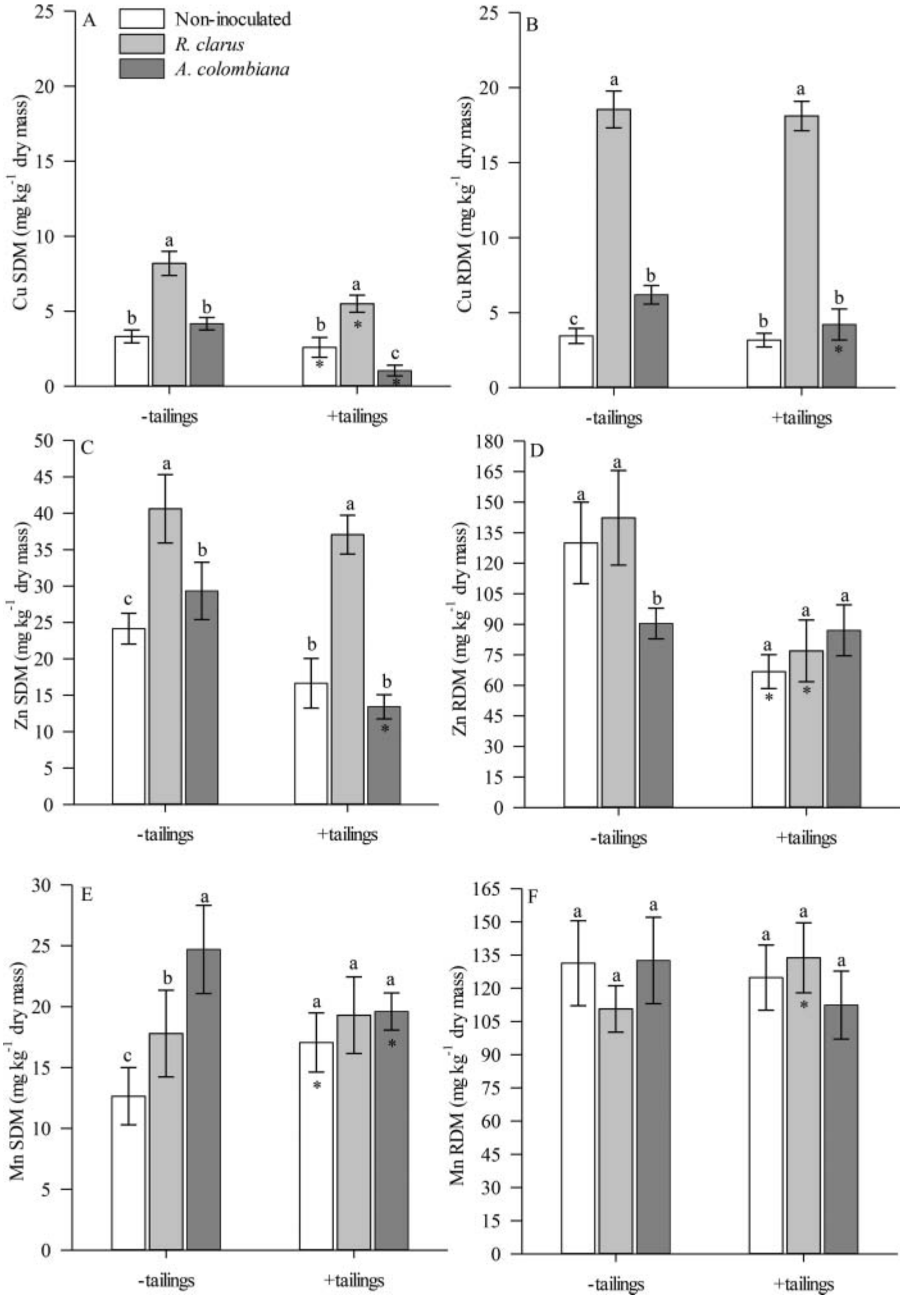

Figure 3. Copper in shoot dry mass (SDM) (A), cooper in root dry mass (RDM) (B), Zn in shoot dry mass (SDM) (C), Zn in root dry mass (RDM) (D), Mn in shoot dry mass (SDM) (E), and Mn in root dry mass (RDM) (F) of pacara earpod tree seedlings, in a greenhouse, in soil with or without coal-mine tailing and non-inoculated or inoculated with either *Rhizophagus clarus* or *Acaulospora colombiana*. [1]Means followed by the same letter are not different in the effect of AMF inoculation within each level of tailings. *Significative effect of tailings within each AMF inoculation treatment (Tukey test, $p < 0.05$, $n = 12$).

substrate (Figure 3a). The results were similar for the roots; root Cu content was 72% higher in *R. clarus*-inoculated seedlings than in *A. colombiana*-inoculated seedlings, regardless of tailing contents (Figure 3b). Copper tissue content of 20 mg kg^{-1} may be phytotoxic to some plant species and impair their growth (Kabata-Pendias 2011). However, in partial tailing substrates, inoculating pacara earpod tree seedlings with *A. colombiana* had a phytoprotective effect against Cu, reducing its uptake in roots and translocation to shoots by one-third (Figure 3).

Zinc shoot contents were about 50% higher in *R. clarus*-inoculated plants than in controls or *A. colombiana*-inoculated seedlings, regardless of the tailing content (Figure 3c). Plants inoculated with *R. clarus* had up to 165 mg kg^{-1} of Zn in the roots (Figure 3d), with no visible toxicity symptoms. Since Zn content above 100 mg kg^{-1} has been linked to phytotoxic effects in some plant species (Kabata-Pendias 2011), it appears that association with AMF has phytoprotective effects on pacara earpod trees. The fact that *A. colombiana* provides phytoprotection against Zn in partial tailing substrate is demonstrated by the 50% reduction in uptake and translocation to shoots, compared to all other treatments (Figure 3). Meyer *et al.* (2016) also observed lower translocation of Zn in vetiver plants inoculated with *A. colombiana* than in those inoculated with *R. clarus*. The lower Zn translocation of seedlings inoculated with *A. colombiana* improved the seedling quality.

Table 3. Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) accumulation in shoot dry matter (SDM) of pacara earpod tree seedlings grown in a greenhouse, in soil with or without coal-mine tailing, and non-inoculated or inoculated with *Rhizophagus clarus* or *Acaulospora colombiana*.

Nutrient	Ni-T	*Rc*-T	*Ac*-T	Ni + T	*Rc* + T	*Ac* + T
			mg plant^{-1}			
N Cv = 25.19%	8.25 a[1]	8.18 a	2.93 a	16.57 b*	15.36 b*	31.19 a*
P Cv = 16.89%	3.77 a	4.75 a	3.52 a	5.41 b*	8.80 a*	6.06 b*
K Cv = 21.39%	2.21 ab	2.79 a	1.73 b	2.65 b	3.81 a*	2.31 b
Ca Cv = 15.66%	2.67 a	2.71 a	1.24 b	5.30 a*	5.38 a*	5.85 a*
Mg Cv = 19.02%	3.18 a	3.80 a	3.27 a	3.39 a	3.86 a	3.16 a

Ni, non-inoculated; *Rc, Rhizophagus clarus*; *Ac, Acaulospora colombiana*; -T = without tailing; +T = with tailing; Cv = coefficient of variation.

[1] Means followed by the same letter on the same line were not different within each level of tailing.

*indicates significant effect of tailing within each level of inoculation (Tukey test, $p < 0.05$, n = 4).

Manganese content was around ten times higher in the roots than in the shoots of all treatment types, and Mn shoot content was higher in seedlings planted in partial tailing substrate than in normal soil, although this content decreased in *A. colombiana*-inoculated seedlings planted in partial tailing substrate (Figure 3e and f). However, translocation of this element was generally lower than that of Cu and Zn (Figure 3). The manganese content was also below levels considered phytotoxic (Kabata-Pendias 2011).

Macronutrients in biomass

Macronutrient accumulation was affected by AMF inoculation, mainly in partial tailing substrate with the highest N shoot accumulations occurring in *A. colombiana*-inoculated seedlings (Table 3), which were approximately 50% higher than in controls or *R. clarus*-inoculated seedlings (Table 3). This could be related to the synergistic effects of the tripartite interaction. Moreira *et al.* (2010) also found no N deficiency when *E. contorstisiliquum* was inoculated with AMF and *B. elkanii* simultaneously in low-fertility soil. *R. clarus*-inoculated seedlings in

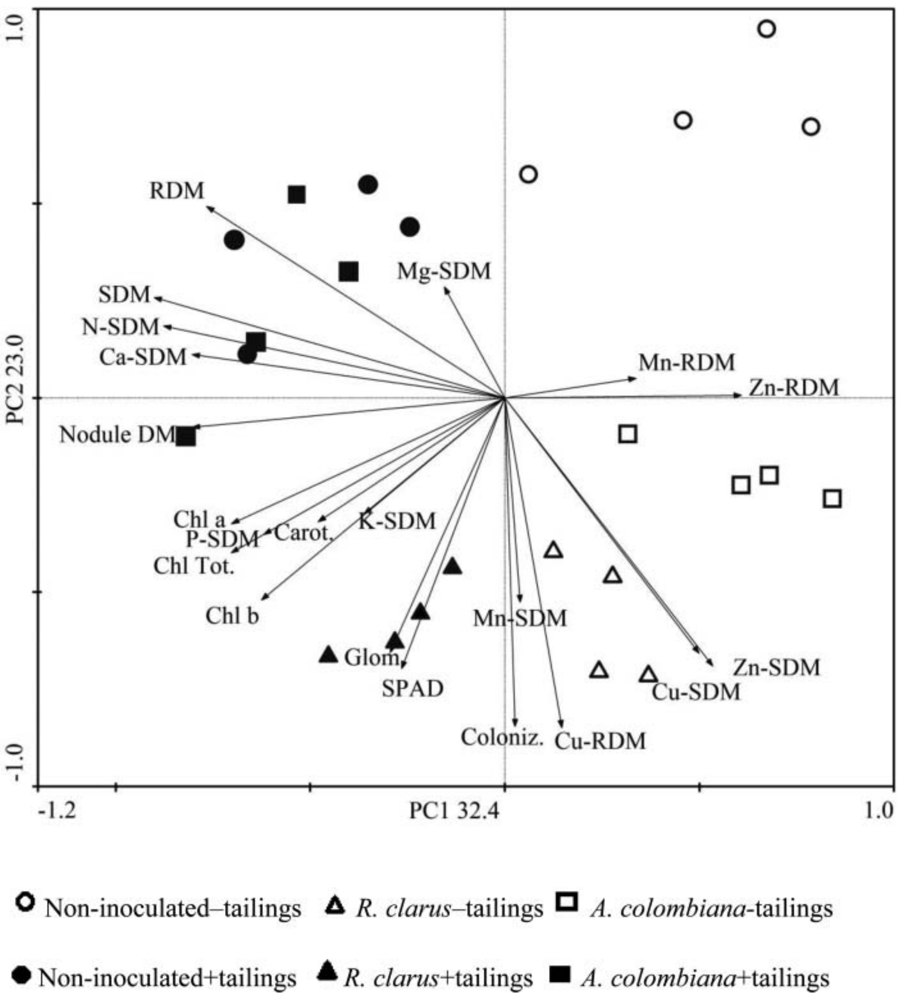

Figure 4. Principal component analysis (PCA) performed with four replicates of each variable analyzed in pacara earpod tree seedlings grown in a greenhouse in soil with or without coal-mine tailing and with or without arbuscular mycorrhizal fungi inoculation. Shoot dry matter (SDM); root dry matter (RDM); SPAD index (SPAD); chlorophyll a (Chl a); chlorophyll b (Chl b); total chlorophyll (Chl Tot.); carotenoids (Carot.); nitrogen in shoots (N-SDM); phosphorus in shoots (P-SDM); potassium in shoots (K-SDM); calcium in shoots (Ca-SDM); magnesium in shoots (Mg-SDM); mycorrhizal colonization (Coloniz.); total glomalin (Glom.); nodule dry mass (Nodule DM); Cu in shoots (Cu-SDM); Cu in roots (Cu-RDM); Zn in shoots (Zn-SDM); Zn in roots (Zn-RDM); Mn in shoots (Mn-SDM), and Mn in roots (Mn-RDM).

partial tailing substrate accumulated 31% and 38% more P in their shoots than *A. colombiana*-inoculated seedlings and controls, respectively (Table 3). This result may be related to the higher percentage of colonization by this fungus (Ferreira *et al.* 2015; Moreira *et al.* 2010; Meyer *et al.* 2016). Similar behavior was observed for K: the highest values were found in *R. clarus*-inoculated seedlings in partial tailing substrate, while association with *A. colombiana* did not increase the accumulation of this nutrient (Table 3).

Mycorrhizal inoculation did not affect Ca content in shoot tissues, although the addition of tailings enhanced the uptake and accumulation of this nutrient (Table 3). Magnesium content in the shoots did not differ among treatments (Table 3).

Principal component analysis

According to PCA, the addition of tailing and inoculation effects accounted for 55.4% of variance in the data. Most of the total variance was concentrated in PC1 (32.4%), and was related to shoot Cu content, root Cu content, shoot Zn concentration, shoot Mn, SPAD index, AMF colonization percentage, and soil glomalin content (Figure 4).

Inoculation with *R. clarus* in partial tailing substrate increased P and K accumulation (Table 3), shoot Cu and Zn content (Figure 3a–d), and was related to levels of these trace elements in shoots and roots (Figure 4). This may be due to higher colonization by this fungal species (Figure 1a). Phosphate can contribute to plant growth in contaminated soils by forming insoluble compounds with heavy metals in the roots, reducing its translocation to shoots (van Steveninc *et al.* 1994). With *A. colombiana* inoculation in partial tailing substrate, P may have remained complexed with Cu and Zn in roots, since there were low total and shoot levels of these elements (Figure 3).

The different phytoprotective effects that these two AMF isolates had on pacara earpod tree seedlings is linked with the different mechanisms mediated by arbuscular mycorrhizae (Smith and Read 2008; Cabral *et al.* 2015; Soares *et al.* 2001; Doubková and Sudová 2016; Ferreira *et al.* 2015; Meyer *et al.* 2016). *A. colombiana* presented lower mycorrhizal colonization and P uptake, but it contributed significantly to plant protection, which underscores the complexity of the mechanisms involved in abiotic stress tolerance in this tree species. The phytoprotective effect of *A. colombiana* can be characterized by its reduction of Cu and Zn uptake in roots and translocation to shoots (Figure 3a–d), as well as by the observed glomalin level increases in the soil (Figure 1b). In addition, *A. colombiana* inoculation contributed to increased nodule weight (Figure 1c) and N accumulation in the shoots (Table 3), which is necessary for chlorophyll synthesis (Table 2). These effects resulted in the higher growth and quality of *A. colombiana*-inoculated seedlings in partial tailing substrate (Figure 2).

Conclusions

The pacara earpod tree has potential for use with re-vegetation programs in soils degraded by coal mining. In addition to its ability to fix nitrogen, this species presents high mycorrhizal colonization with consequent improvement in P uptake, especially when inoculated with *R. clarus*. The pacara earpod tree's Cu and Zn phytoextractive potential was verified when inoculated with *R. clarus*: its association with this FMA has potential for use in phytoextraction programs. Inoculation with *A. colombiana*, however, had phytoprotective effects on this tree species in soil containing coal-mining waste, reducing its uptake and translocation of Cu and Zn and promoting soil glomalin production.

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