Rhizophagus Clarus and Phosphorus in Crotalaria juncea: Growth, Glomalin Content and Acid Phosphatase Activity in a Copper-Contaminated Soil

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ABSTRACT: *Crotalaria juncea* is used as plant cover in grape vineyards in Brazil, which usually present soils with high copper (Cu) levels due to the application of Cu-based phyto-sanitary products. Under this condition an increase growth and cover of *C. juncea* is needed to improve the phytoremediation processes in those soils. Some alternatives to achieve this condition is the inoculation with arbuscular mycorrhizal fungi (AMF), which has demonstrated an important increase of plant growth in Cu-contaminated soils at different soil P levels. The aims of this study were to evaluate the effect of AMF inoculation in soils with high Cu contents on the growth of *C. juncea*, the acid phosphatase (APase) enzyme activity in plants and soil, and the presence of glomalin under different P supply conditions, as a basis to identify if there is a synergistic interaction between AMF inoculation and P supply on soils with high Cu levels. The experiment was carried under greenhouse conditions in a factorial 3 × 2 design (natural P content, addition of 40 and 100 mg kg^{-1} P, with and without the inoculation of the AMF *Rhizophagus clarus* with three replicates) in a soil with high Cu content (60 mg kg^{-1}). The addition of 40 and 100 mg kg^{-1} P favored plant growth both in the presence and in the absence of AMF. However, when plants were grown in soil with a natural P level, the inoculation with AMF increased by 116 % the shoot biomass, compared to the non-inoculated treatment. Our results showed that the combination of P supply and *R. clarus* inoculation could be an adequate strategy to reduce Cu phytotoxicity in *C. juncea*, as it increases plant biomass and modify the APase enzyme activity in the soil and plant. Additionally, glomalin produced by the AMF and accumulated in the soil can decrease the availability of Cu to the plants by means of sequestration beyond the root surface, with a consequent plant protective effect.

Keywords: phytoremediation, leguminous, FMA, vineyards.

INTRODUCTION

Grape vines in Brazil are cultivated in humid areas, which favor the presence of fungal foliar diseases, limiting the productivity of grapes. For this reason, vines annually undergo successive treatments with copper (Cu)-based foliar fungicides (Mackie et al., 2012), which cause an important Cu accumulation in the soils over time (Casali et al., 2008; Nogueirol et al., 2010). High Cu contents in superficial soil layers reduce the establishment and growth of spontaneous or introduced herbaceous plants, which are generally growing between the vines (Panou-Filotheou et al., 2001). Additionally, some fractions of the total Cu can be present in the soil solution or adsorbed to charged colloidal particles and may thus be able to reach the surface waters adjacent to the vineyards (Karathanasis, 1999). Moreover, some of the Cu may migrate through the soil profile, especially in sandy soils, thereby increasing the potential for contamination of subsurface waters (Fernández-Calviño et al., 2012).

Throughout the lifecycle of the vines, or even after the eradication of the vineyards, cover crop species, such as *Crotalaria juncea* L. (Fabaceae) can be cultivated to increase soil coverage, contributing to increase the organic residue in the soil. The growth of *C. juncea* can be favored by the establishment of root symbionts, such as those with arbuscular mycorrhizal fungi (AMF), but little is known regarding the role of these organisms in the protection of plants against an excess of potentially toxic elements (PTE), such as Cu, or the subsequent effects on biomass production and other physiological parameters. The plant protective effects of AMF have been previously observed for different grass (Soares and Siqueira, 2008) and tree (Treseder and Vitousek, 2001) species. However, it has been suggested that AMF may protect plants against an excess PTE, possibly through the following mechanisms: i) the dilution of PTE in plant tissue due to the increase in plant growth (Christie et al., 2004); ii) the prevention of absorption through the precipitation or chelation of the elements in the rhizosphere (Kaldorf et al., 1999; Meier et al., 2012a); and iii) the reduction of PTE absorption due to the sequestration and immobilization in the fungal structures, and iv) the production of glomalin, a glycoprotein produced by AMF (Khan et al., 2000; Zhu et al., 2001; González-Chávez et al., 2002; Cornejo et al., 2008; Aguilera et al., 2011; Meier et al., 2012b; Cornejo et al., 2013), with a consequent reduction in the transport of elements from the roots to shoots (Joner et al., 2000; Christie et al., 2004). These effects of AMF on their host plants vary depending on the fungal isolates tested and the soil contaminants (Silva et al., 2006), which may be related to the PTE retention capacity by the fungal mycelium.

Previous studies done *in vitro* with the AMF *Rhizophagus clarus* (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler have demonstrated a differentiated capacity of the fungus to retain Cu, Zn, Cd, and Pb in the mycelium (Cabral et al., 2010). This behavior was attributed to the differential production of glomalin, however the role of this protein has not been experimentally evaluated in tropical soils containing excessive levels of PTE. As the AMF act as protective agents for the plants and can also favor the extraction of PTE from the soil (Christie et al., 2004), these fungi can perform an important role in the plant tolerance to these contaminants, as most of the plant species form mycorrhizal symbioses, even in highly contaminated areas (Klauberg-Filho et al., 2005; Meier et al., 2012b).

On the other hand, the AMF can also reduce the phytotoxicity of PTE due to the increase in phosphorus (P) acquisition by the host plant (Soares and Siqueira, 2008; Rangel et al., 2014). Some studies indicate that an adequate supply of P also allows an increase in PTE retention by plant roots, thus restricting the translocation of such elements to the shoots (Soares et al., 2006). This phenomenon occurs because the metal can form stable complexes with P, thus being retained in the roots (van Steveninck et al., 1994). In addition, the increase in soil P contents, derived from the long fertilization cycles for the vines, may reduce the availability of Cu in the soil solution by forming insoluble

Cu-phosphates (Ayati and Madsen, 2000; Cao et al., 2003). The phosphate anion can bind to the surface of reactive particles of soil, such as oxides, thus increasing negative charges (Barrow, 1999; Pérez-Novo et al., 2009), which increases the potential for the formation of oxide-phosphate-metal ternary complexes (McBride, 1994). These, in turn, reduce the availability of Cu in the soil solution and, consequently, its phytotoxicity. Due to the P-metal interaction, there is a decrease in P availability in the soil solution with a consequent impairment of P absorption by plants. Therefore, some plant and microorganism species have a strategy of increasing phosphatase activity in their roots in response to a P deficiency in the soil, showing its importance for plant nutrition (Nuruzzaman et al., 2006; Tabaldi et al., 2011; Wang et al., 2013). However, this aspect has not been well investigated in soils containing excess metals in tropical conditions, which can be an indirect mechanism to reduce the availability of Cu increasing the plant tolerance in a potential phytoremediation framework.

The objectives of this study were to evaluate the effect of P application and AMF inoculation in soils with high Cu content by measuring the growth of *C. juncea*, the acid phosphatase enzyme activity in plants and soil, and the presence of glomalin (as glomalin-related soil protein - GRSP).

MATERIALS AND METHODS

Experimental design

The study was conducted in a greenhouse of the Department of Soil Science of the Federal University of Santa Maria (*Universidade Federal de Santa Maria* - UFSM). A 3×2 factorial design was employed in a completely randomized scheme with three replicates. The treatments consisted of three P levels: i) natural level (5.6 mg kg^{-1}); natural plus the supply of ii) 40 or iii) 100 mg kg^{-1} P. These levels of P were determined according to previous studies examining P and heavy metal interactions in similar soils (Soares and Siqueira, 2008). Phosphorous was applied as triple superphosphate to a soil artificially contaminated by adding 60 mg kg^{-1} Cu. This level of Cu is commonly observed in vineyard soils in the Campanha Gaúcha, Brazil (Miotto et al., 2014). The soils of each P level were inoculated (+AMF) with spores of *Rhizophagus clarus*, as further described, or maintained as non-inoculated (-AMF) soil.

Substrate preparation and soil analyses

A Typic Hapludalf (*Argissolo Vermelho distrófico*) soil with a sandy texture (87 % and 8 % of sand and clay, respectively) was collected from a natural grassland area (30° 48' 13.03" S; 55° 23' 5.48" W). The soil pH was adjusted to 6.0 via lime addition, then supplemented with 40 or 100 mg kg^{-1} P and allowed to stabilize for 45 days. Subsequently, the soil was contaminated with 60 mg kg^{-1} Cu (CuSO$_4$.2H$_2$O) and allowed to stabilize for another 45 days. Finally, the soil was autoclaved twice at 120 °C for two hours. A basal fertilization of 100, 30, 5, and 0.80 mg kg^{-1} of N (NH$_4$Cl), K (K$_2$SO$_4$), Zn (ZnSO$_4$.7H$_2$O) and B (H$_3$BO$_3$), respectively, was applied to all plots. Nitrogen fertilization was divided between two applications, delivered at 15 and 30 days after germination.

Biological material

The *Crotalaria juncea* seeds were scarified with concentrated H$_2$SO$_4$ for 5 min and then rinsed in distilled autoclaved water. Four seeds were sown in each pot, and 10 days later only two plantlets were kept in each pot. The inoculation with AMF was performed using 200 spores of *R. clarus* (isolate # UFSC-14) per pot, provided by the Soil Microbiology Laboratory of the Federal University of Santa Catarina, Santa Catarina State, Brazil, after multiplication in pots with soil cultivated with *Brachiaria decumbens*. The spores were extracted by the method of wet sieving and decanting (Gerdemann and Nicolson,

1963) and later by centrifugation in water at 700 g for three minutes, and sucrose (45 %) at 300 g for two minutes. The counting was done by using a stereomicroscope (40 X). In the non-inoculated treatments, 50 mL of a suspension obtained by mixing 100 g of soil in 1 L of sterile water filtered in a 45 μm sieve was applied. This FMA-free suspension was used to balance the edaphic microbiota of the non-inoculated treatments.

Plant and soil analyses

After incubation and prior to seeding, a soil solution was extracted to assess the effects of P addition on the chemical species of Cu and P. Thereunto, the soil solution was then collected in a saturation extract following the methodology described by van Raij et al. (2001). The pH was determined in an aliquot of soil solution. Another aliquot was then filtered using a 0.22 mm cellulose membrane filter. The total soluble organic carbon (SOC) concentration was determined according to Silva (2001). Total cation concentrations in the soil solution (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, K^+, and Na^+) and total P content were determined by using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Perkin Elmer Optima 7000DV); the anions content (NO_3^-, SO_4^{-2}, and Cl^-) were determined by using high performance liquid chromatography (HPLC). The ionic speciation of the soil solution was performed by using Visual MINTEQ software version 2.15 (Gustafsson, 2004).

The levels of available Cu and Zn in the soil were determined after extraction with an EDTA solution (0.01 mol L^{-1} ethylenediamine tetraacetic acid, 1 mol L^{-1} ammonium acetate, pH 7.0), and the available Ca and Mg in the soil were extracted with 1 mol L^{-1} KCl. The available P and K were extracted from 5 g of dry soil with 50 mL of Mehlich-1. The main soil chemical properties after the conditioning process are listed in table 1.

After 45 days of cultivation, the dry mass yield of shoots (SDM) and roots (RDM) were evaluated, and the plant's rhizosphere collected to analyze the activity of acid phosphatase in the soil and the total (T) and easily extractable (EE) glomalin-related soil protein (GRSP) content. The plants were harvested, cutting the shoots close to the soil, and the material gathered was rinsed in distilled water. The roots were rinsed in running water until the soil was completely removed, then rinsed with a 0.1 mol L^{-1} HCl solution and, finally, rinsed in distilled water. The roots were separated from the soil, and approximately 1.0 g was collected to evaluate the mycorrhizal colonization. The aerial portion and the roots were dried in an oven with forced air circulation at 60-70 °C until a constant mass was achieved.

Total P and Cu contents in shoots and roots were determined using ICP-OES following an HNO_3-$HClO_4$ digestion (Ferreira et al., 2013). To quantify the acid phosphatase in the shoots, the fourth expanded leaf from the upper third portion of each plant was collected, immediately frozen in liquid N_2, and stored at -80 °C. Later, 1.0 g of each sample was macerated in liquid N_2 and homogenized in 3.0 mL of 100 mmol L^{-1} Tris-HCl buffer (pH 7.4), 1.0 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), and 0.1 % albumin. The mixture was then centrifuged at 20,000 g for 30 min, and the resulting supernatant was used in the enzyme assay. The acid phosphatase (APase) activity in the plant material was determined following the method of Tabaldi et al. (2007), using a reaction medium that consisted of 3.5 mmol L^{-1} sodium azide, 2.5 mmol L^{-1} calcium chloride, and 100 mmol L^{-1} citrate buffer (pH 5.5), for a final volume of 200 μL. An aliquot of 20 μL of the sample was added to the reaction mixture, with the exception of the control, and incubated for 10 min at 35 °C. The reaction was triggered by adding 3.0 mmol L^{-1} inorganic pyrophosphate (PPi) and halted after 10 min by adding 200 μL of 10 % trichloroacetic acid (TCA).

Acid phosphatase activity in the soil was estimated by measuring the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl phosphate following the exposure of the soil to a modified universal buffer (MUB) at pH 6.5, following the method of Tabatabai and Bremner (1969). The soil samples (1.0 g) were incubated with 1.0 mL of *p*-nitrophenyl phosphate 0.1 mol L^{-1} and 4.0 mL of MUB for 60 min at 37 °C. At the end of the incubation period,

Table 1. Chemical properties of the soil, soil solution, and ionic speciation after treatment with P and Cu

Soil chemical characterization	Natural P	40 mg kg^{-1} P	100 mg kg^{-1} P
pH(H$_2$O)	5.9	5.6	5.5
C (g kg^{-1})	6.5	6.5	6.5
Available Cu by EDTA (mg kg^{-1})	45.6	45.5	42.5
Available P by Mehlich-1 (mg kg^{-1})	5.6	34.1	85.3
Available K by Mehlich-1 (mg kg^{-1})	190.5	183.5	170.5
Exchangeable Ca (mg kg^{-1})	458.4	458.4	552.8
Exchangeable Mg (mg kg^{-1})	90.7	94.1	94.4
Soil solution chemical characterization			
pH	4.9	4.9	4.8
Na (mg L^{-1})	12	18	13
Al (mg L^{-1})	4.0	3.4	4.2
K (mg L^{-1})	21	25	28
Mg (mg L^{-1})	126	133	135
Ca (mg L^{-1})	266	286	332
Cu (mg L^{-1})	10.5	7.3	4.8
Fe (mg L^{-1})	1.9	1.4	1.2
Zn (mg L^{-1})	0.8	0.5	0.4
Cl (mg L^{-1})	108	119	138
SO$_4^{2-}$ (mg L^{-1})	86	102	126
NO$_3^-$ (mg L^{-1})	265	314	310
PO$_4^{3-}$ (mg L^{-1})	5.2	7.4	10.1
Soluble organic carbon (mg L^{-1})	654	669	635
Chemical speciation of Cu in the soil solution (%)			
Cu^{2+}	2.7	2.8	3.4
Cu-DOM	97.0	96.0	96.0
CuCl$_2^-$	0.0	0.0	0.0
CuCl$_{(aq)}$	0.0	0.0	0.0
Chemical speciation of P in the soil solution (%)			
H$_2$PO$_4^-$	77.0	81.0	79.0
AlHPO$_4^+$	15.0	11.0	13.0
HPO$_4^{2-}$	0.0	0.0	0.0
CaH$_2$PO$_4^+$	0.0	0.0	0.0

1.0 mL of CaCl$_2$ 0.5 mol L^{-1} and 4.0 mL of NaOH 0.5 mol L^{-1} were added. The solution was then quickly filtered with a Whatman 2 v filter. The samples were homogenized, and the concentration of PNP formed was determined at 400 nm using a spectrophotometer.

The quantification of EE-GRSP and T-GRSP was performed according to Wright and Upadhyaya (1998). To quantify EE-GRSP, 1.0 g of air-dried rhizosphere soil was used. The extraction was performed with 8.0 mL of 20 mmol L^{-1} sodium citrate at pH 7.0 for 30 min at 121 °C. The T-GRSP was extracted with 50 mmol L^{-1} sodium citrate at pH 7.0 after three 1-hour autoclaving cycles at 121 °C. The supernatant was separated from the soil through centrifugation at 1,000 *g* for 10 min. The protein in the supernatant was quantified using the Bradford assay (1976), with bovine serum albumin (BSA) as standard (Wright et al., 1996). Both EE and T-GRSP concentrations were corrected to mg g^{-1} considering the dry soil mass and the total supernatant volume.

The collected roots were stored in FAA solution [formaldehyde (40 %): alcohol (50 %): glacial acetic acid = 13:200:5 mL] and later cleared and stained using the Phillips and Hayman method (1970). The colonization rate was evaluated on a grid plate (Giovannetti and Mosse, 1980). The number of AMF spores in the soil was determined by wet sieving and centrifugation in a sucrose solution (Gerdemann and Nicolson, 1963) using 50 mL of soil from each experimental unit.

Statistical analysis

All the data were transformed when necessary to meet the assumptions of normality and homoscedasticity. Subsequently, the data were analyzed by Anova, and the means compared using Tukey's test when the effects of AMF inoculation, P supply, and/or the interaction between these factors were statistically significant (p<0.05). Moreover, the linear correlations (Pearson's correlation coefficients) among the data were determined using the SigmaPlot version 12.3 software. In addition, the data from the variables that presented the highest correlation were submitted to the analysis of Detrended Correspondence Analysis (DCA), in which it was found that there was a linear distribution and, therefore, Principal Component Analysis (PCA) was performed. The software Canoco version 4.5 (Ter Braak and Smilauer, 2002) was used for the DCA and PCA after data standardization.

RESULTS

Chemical speciation of the soil solution

Phosphorous supply reduced the availability and altered the chemical species of Cu in the soil solution; moreover, the addition of Cu caused a decrease in pH (Table 1). Independent of the amount of P added to the soil, most of the Cu was bounded to dissolved organic carbon (Cu-DOM = 96 %) and only 3 % was in the form of free Cu^{2+}. Furthermore, the addition of 100 mg kg^{-1} P reduced in 54 % the Cu concentration in the soil solution in relation to the treatment with natural P content, showing an inverse relationship between P supplied and Cu in solution. As the concentration of P increased in the soil solution, the concentrations of both Fe and Zn in the solution decreased. The PO_4^{3-} concentration in the soil solutions among the treatments varied from 5.20 to 10.10 mg L^{-1} (Table 1), and the P form predominant in the soil solution was $H_2PO_4^-$, leading to a soil solution pH of 4.8, on average. The addition of 60 mg kg^{-1} Cu also changed the availability of NO_3^-, Ca^{2+}, Mg^{2+}, and K^+ in the soil solution.
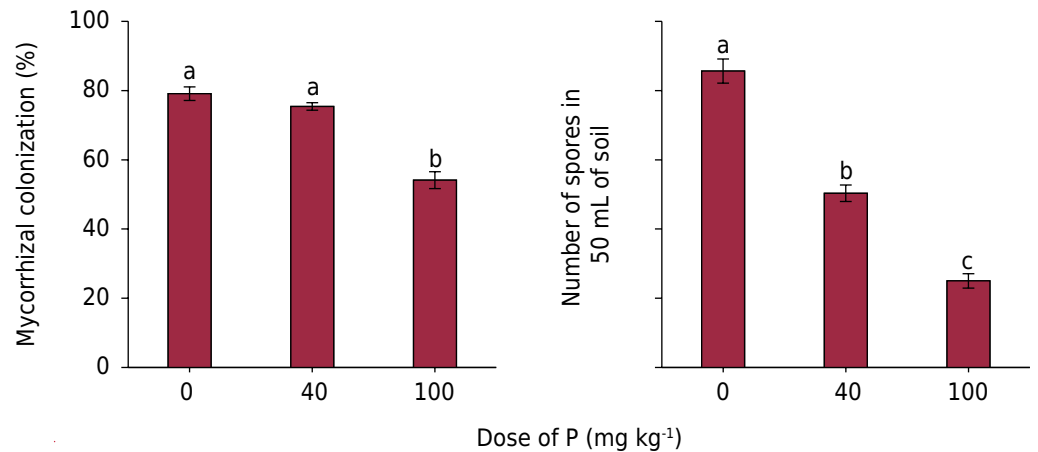
Mycorrhizal colonization, sporulation, and glomalin concentration

None of the treatments without AMF inoculation had evidence of spores or AMF colonization. However, with the AMF inoculation, the colonization did not differ between soils with natural P content and those with 40 mg kg^{-1} P (76-80 %), but colonization was lower with the addition of 100 mg kg^{-1} P (54 %; Figure 1). Additionally, the number of AMF spores in the soil decreased proportionally with the increase of P, from 86 to 25 spores in 50 mL of soil (Figure 1).

The EE-GRSP and T-GRSP contents were both greater in the treatments with the AMF inoculation (Figure 2). In the soils with natural P content the E-GRSP content was 1.56 times higher in the presence of AMF, but for T-GRSP the inoculation increased the contents by 125 % compared with the non-inoculated treatment.

Plant growth and Cu and P absorption by *C. juncea*

The addition of 40 and 100 mg kg^{-1} P favored plant growth both in the presence or in the absence of AMF (Figure 3). However, when the plants were grown in the soil with natural P content, the inoculation with AMF increased by 116 % the SDM yield, compared to the

Figure 1. Mycorrhizal colonization and number of spores of *Crotalaria juncea* grown in a soil with high copper (60 mg kg^{-1}) levels under different levels of phosphorus with and without AMF inoculation. Vertical bars above the columns indicate the standard deviation. Means followed by the same lowercase letter compare doses of P (Tukey 5 %).
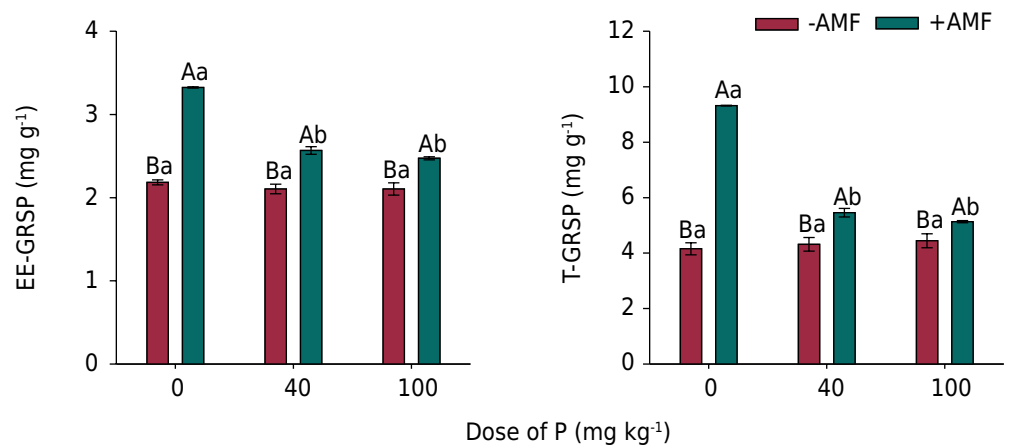
Figure 2. Easily extractable (EE) and total (T) glomalin-related soil protein (GRSP) in rhizosphere soil of *Crotalaria juncea* plants growing at high copper (60 mg kg^{-1}) levels under different levels of phosphorus with and without AMF inoculation. Vertical bars above the columns indicate the standard deviation. Means followed by the same lowercase letter compare doses in the same condition inoculation and uppercase letters compare inoculation within the same dose of P (Tukey 5 %).
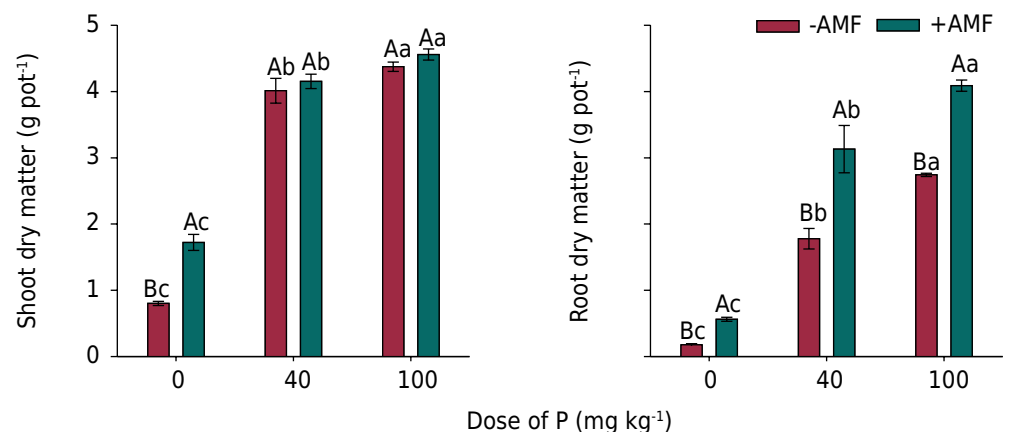
Figure 3. Shoot and root dry matter of *Crotalaria juncea* plants growing in a soil with high copper (60 mg kg^{-1}) levels under different levels of phosphorus with and without AMF inoculation. Vertical bars above the columns indicate the standard deviation. Means followed by the same lowercase letter compare doses in the same condition inoculation and uppercase letters compare inoculation within the same dose of P (Tukey 5 %).

non-inoculated treatment. In the treatments with the addition of 40 and 100 mg kg^{-1} P, the AMF inoculation had low influence on SDM yield. The RDM yield in the treatment with natural P contents was 0.17 and 0.56 g per pot for the inoculated and non-inoculated treatments, respectively (Figure 3). The RDM yield increased with the increase of P in the soil, both with and without AMF inoculation. In the treatments with the addition of 40 and 100 mg kg^{-1} P, there was a greater RDM yield of plant in the presence and absence of AMF when compared with treatments with natural P content in the non-inoculated soil. On the contrary to that observed for the shoots, it was found that the RDM yield was favored both by AMF inoculation and the increase of P, demonstrating a complementing effect of P supply and AMF inoculation in the growth of *C. juncea* roots.

In the treatment with no addition of P, the shoot P content of plants inoculated with AMF was 1,638 mg kg^{-1} versus 724 mg kg^{-1} for the treatment without inoculation (Figure 4). The root P content ranged from 1,141 to 2,177 mg kg^{-1} P when inoculated with AMF, and from 636 to 2,154 mg kg^{-1} when non-inoculated. The root P contents of treatments with natural P levels were 636 and 1,141 mg kg^{-1}, with and without AMF inoculation, respectively. With increasing quantities of P applied to the soil, the contents of P in the roots increased significantly, regardless of inoculation.

In the soil with the natural content of P, the inoculation with AMF resulted in a significant decrease in the Cu content in the shoots, and an increased Cu content in the roots (Figure 4). In the treatments with natural P content, the shoot Cu contents were 79 and 45 mg kg^{-1} with and without AMF inoculation, respectively; whereas, the shoot Cu contents were not influenced by the inoculation when 100 mg kg^{-1} P was added. In contrast, when plants were inoculated with AMF the root Cu contents were increased.

Acid phosphatase enzyme activity in soil and leaves.

The soil APase activity ranged from 144 to 269 µg g^{-1} h^{-1} PNF in plants inoculated with AMF and from 140 to 244 µg g^{-1} h^{-1} PNF in the non-inoculated plants (Figure 5). The soil APase activity in the treatment with 40 mg kg^{-1} P in the AMF inoculated treatment decreased 32 % compared to treatment without AMF inoculation.

The APase activity in *C. juncea* leaves was lower with AMF inoculation (Figure 5). Without inoculation, the APase activity in leaves was higher in the treatments in the soils with the natural P content (Figure 5). By adding 40 mg kg^{-1} P, the values observed were 335 U mg^{-1} protein in plants inoculated with AMF, and 439 U mg^{-1} protein in the non-inoculated plants. However, the APase activity in leaves was not influenced by the amount of P supplied to the soil when inoculated with AMF (Figure 5). The APase activity in the leaves was greater in the soil with natural P levels and without inoculation with AMF than in the soil with the addition of 40 and 100 mg kg^{-1} of P either inoculated or non-inoculated.

Multiple relationships among the studied variables

There were several strong relationships among parameters (Table 2). Moreover, factorial analysis with principal component (PC) extraction showed that PC1 and PC2 explained 81 % of the total experimental variability (46.5 and 33.4 %, respectively; Figure 6). The PC1 was principally correlated with the shoot and root biomass, P content in roots, and PO_4^{3-} contents in soil. On the other hand, the treatments supplied with 100 mg kg^{-1} P, irrespective of the presence of AMF, were highly associated with an increase in the above-mentioned variables. Conversely, the treatments with natural soil P levels showed a greater association with the variables APase activity in leaves, Cu content in shoot, and Cu^{2+} in the soil solution. The experimental units that were non-inoculated and supplemented with 40 mg kg^{-1} P tended to be positioned at an intermediate position in the PCA plot, which indicates a weak effect of those variables.
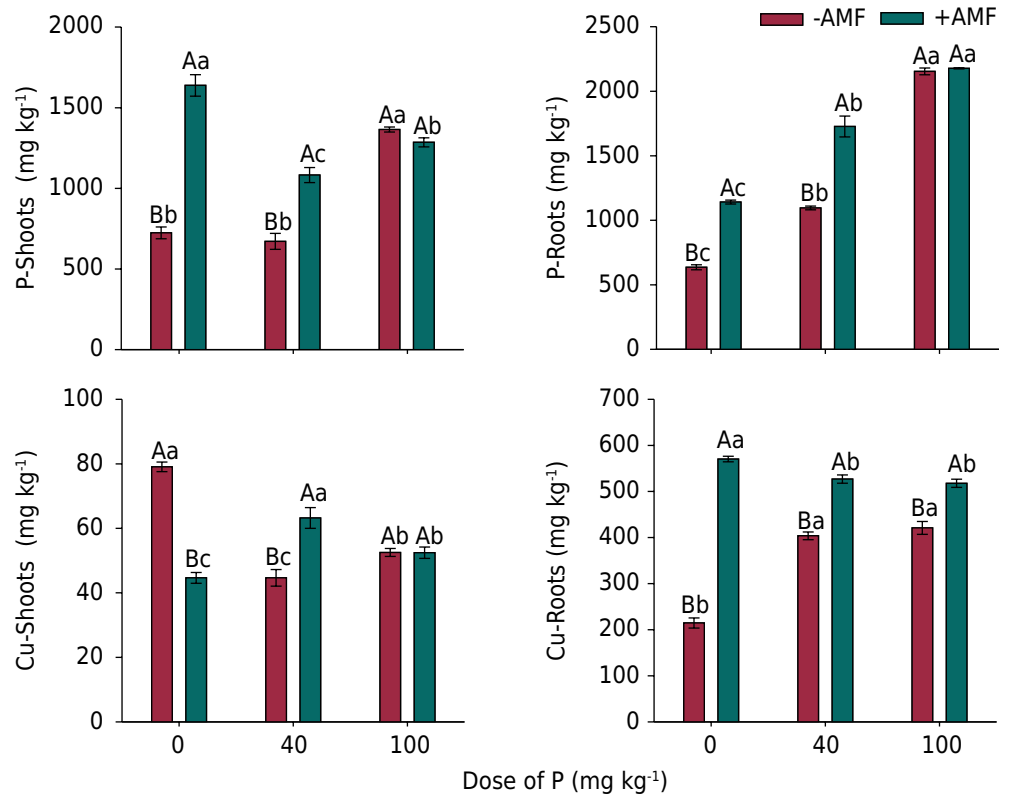
Figure 4. Phosphorus and Cu content in shoots and roots of *Crotalaria juncea* plants growing in a soil with high copper (60 mg kg^{-1}) levels under different levels of phosphorus with and without AMF inoculation. Vertical bars above the columns indicate the standard deviation. Means followed by the same lowercase letter compare doses in the same condition inoculation and uppercase letters compare inoculation within the same dose of P (Tukey 5 %).
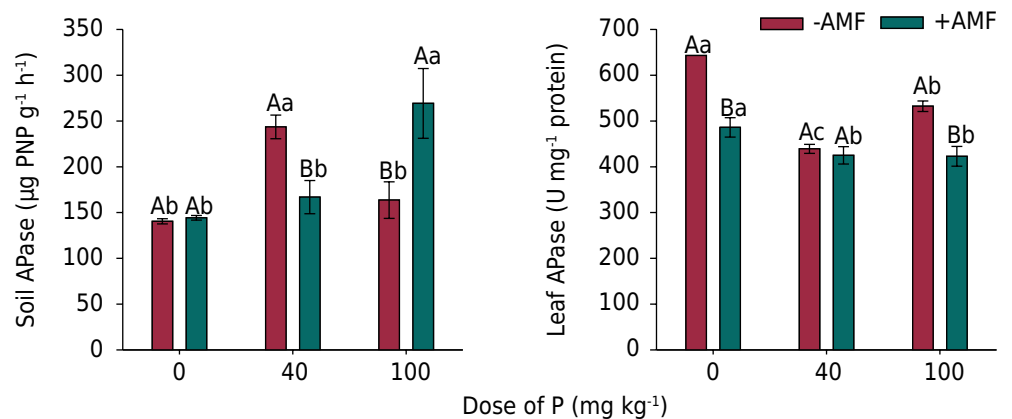
Figure 5. Enzymatic activity of soil and leaf acid phosphatase (APase) in *Crotalaria juncea* plants growing in a soil with high copper (60 mg kg^{-1}) levels under different phosphorus levels with and without AMF inoculation. Vertical bars above the columns indicate the standard deviation. Means followed by the same lowercase letter compare doses in the same condition inoculation and uppercase letters compare inoculation within the same dose of P (Tukey 5 %).

DISCUSSION

Strategies to reduce the solubility and bioavailability of PTE in the soil have been proposed to reduce the impact of such contamination on plant species. In this context, the present study evaluated the effect of P addition and AMF inoculation on the Cu availability in soils with high Cu levels, and their combined effects on the growth and Cu uptake by *C. juncea*. It was demonstrated that the application of P leads to a decline in the Cu availability in the soil solution (Table 1) therefore, facilitating plant growth (Figure 3). As

Table 2. Correlation matrix of some-selected experimental variables

	MC	Spores	SDM	RDM	P-S	P-R	Cu-S	Cu-R	SAP	LAP	T-GRSP	EE-GRSP	Cu²⁺
Spores	0.92***												
SDM	0.04 ns	-0.20 ns											
RDM	0.22 ns	-0.11 ns	0.90***										
P-S	0.61**	0.67**	0.10 ns	0.18 ns									
P-R	0.22 ns	0.00 ns	0.82***	0.88***	0.50*								
Cu-S	-0.22 ns	-0.29 ns	-0.46 ns	-0.26 ns	-0.43 ns	-0.32 ns							
Cu-R	0.81***	0.75***	0.47*	0.47*	0.71***	0.55*	-0.66**						
SAP	-0.06 ns	-0.25 ns	0.58*	0.54*	-0.18 ns	0.36 ns	-0.34 ns	0.21 ns					
LAP	-0.52*	-0.36 ns	-0.72***	-0.64**	-0.24 ns	-0.51*	0.61**	-0.79***	-0.59*				
T-GRSP	0.74***	0.92***	-0.33 ns	-0.29 ns	0.72***	-0.11 ns	-0.42 ns	0.66**	-0.30 ns	-0.20 ns			
EE-GRSP	0.84***	0.96***	-0.32 ns	-0.22 ns	0.69**	-0.08 ns	-0.31 ns	0.67**	-0.24 ns	-0.23 ns	0.97***		
Cu²⁺	0.14 ns	0.38 ns	-0.89***	-0.88***	-0.17 ns	-0.90***	0.31 ns	-0.26 ns	-0.53*	0.43 ns	0.44 ns	0.44 ns	
PO₄³⁻	-0.15 ns	-0.38 ns	0.86***	0.87***	0.21 ns	0.91***	-0.30 ns	0.25 ns	0.50*	-0.39 ns	-0.42 ns	-0.42 ns	-1.00***

MC = Mycorrhizal colonization (%); Spores = number of spores in 50 mL of soil; SDM = shoot biomass (g per pot); RDM = root biomass (g per pot); P-S = P-Shoot (mg kg⁻¹); P-R = P-Root (mg kg⁻¹); Cu-S = Cu-Shoot (mg kg⁻¹); Cu-R = Cu-root (mg kg⁻¹); SAP = soil acid phosphatase (µg g⁻¹ h⁻¹ PNP); LAP = leaf acid phosphatase (U mg⁻¹ protein); T-GRSP = total glomalin-related soil protein (mg g⁻¹); EE-GRSP = easily extractable glomalin-related soil protein (mg g⁻¹); Cu²⁺ = Cu in the soil solution (mg L⁻¹); PO₄³⁻ = P in the soil solution (mg L⁻¹). *, **, ***, and ns: significant at 5, 1, and 0.1 % probability and no significant differences, respectively

- ▲ 0 mg kg⁻¹ P with AMP
- ■ 40 mg kg⁻¹ P with AMP
- ● 100 mg kg⁻¹ P with AMP
- ▲ 0 mg kg⁻¹ P without AMP
- ■ 40 mg kg⁻¹ P without AMP
- ● 100 mg kg⁻¹ P without AMP
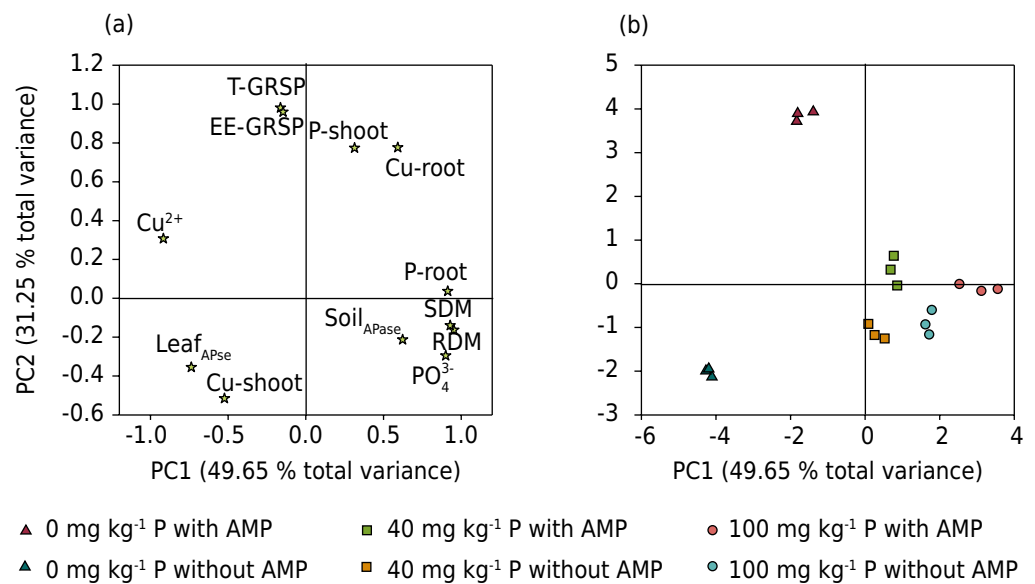
Figure 6. Factorial analysis of the (a) studied variables, and (b) distribution of experimental units according the principal components (PC) obtained, for plants and rhizosphere of *Crotalaria juncea* growing in a soil with high Cu levels. The PC1 and PC2 account for a 79.8 % of the total experimental variance. Conventions: shoot dry matter (SDM), root dry matter (RDM), GRSP$_T$ (total glomalin-related soil protein), and GRSP$_{EE}$ (easily extractable glomalin-related soil protein) for *Crotalaria juncea* grown in a soil with high copper (60 mg kg⁻¹) levels under different levels of phosphorus with and without AMF inoculation.

demonstrated by Ferreira et al. (2015) in a related study, this double strategy including P supply and AMF inoculation on *C. juncea* leads to a better physiological fitness of the plant, which was principally reflected as an activation of a series of anti-oxidant mechanisms. Anti-oxidative mechanisms include increased production of the enzymes such as SOD (superoxide dismutase) and peroxidase (POD), which are responsible for degrading and/or removing reactive oxygen species (O₂⁻, H₂O₂, and OH⁻) present in plants under stress (Mittler, 2002).

In other plant species, this behavior has been also described, representing the AMF colonization a crucial strategy for the plant survival at high Cu levels, also by means of the activation of a series of antioxidant responses (Meier et al., 2011), as well as by a better nutritional status (Meier et al., 2015). This is noticeable, since the Cu has a well-known oxidative effect at membrane and cytoplasmic levels (Meier et al., 2012a; Cornejo et al., 2013). Indeed, the increase of P in the soil solution was positively correlated with SDM yield (r: 0.86; p<0.001), whereas a low P content and high Cu content in the soil solution was negatively correlated with that variable (r: -0.89; p<0.001). The explanation for this phenomenon is that the addition of phosphates is an effective method to remediate soils contaminated with PTE (Cao et al., 2002, 2003; Kede et al., 2008), because of the precipitation of some soluble metals into insoluble mineral species (Ayati and Madsen, 2000; Cao et al., 2003). Additionally, phosphate anions can bind to the surface of reactive particles as oxides, generating a net negative charge (Barrow, 1999), thus allowing the formation of ternary oxide-phosphate-metal complexes (McBride, 1994; Pérez-Novo et al., 2009), which reduce the availability of the metal, such as Cu, in the soil solution (Table 1). However, the use of phosphates as a technology for remediate PTE contaminated soils need to be used as a part of a more complex environmental design, since the availability of natural P sources is limited, and accompanied by an explosive increase in prices (Cordell and White, 2011). Then, other bio-technologies as the use of microbial symbionts must be considered (Meier et al., 2012b).

The growth of *C. juncea* was strongly reduced in the treatments with the natural content of P and without AMF inoculation, compared with those AMF inoculated treatments (Figure 3). This result was related to the increase in P absorption due to a greater volume of soil explored mediated by the AMF (Moreira and Siqueira, 2006). Moreover, there was a greater activity of APase enzymes in the rhizosphere of the treatments with the natural content of P in the absence of AMF (Figure 5). In this situation, the plants, by means of an alteration in their genetic expression, can promote a greater exudation of APase to the soil (Raghothama and Karthikeyan, 2005), increasing the dephosphorylation of organic compounds and providing inorganic P to maintain the cellular metabolism of meristematic root zones (Yun and Kaeppler, 2001; Nanamori et al., 2004; Bozzo et al., 2006). In addition, the lower production of root dry mass in the treatments with the natural content of P and excess Cu might be a result of the Cu toxicity, since the amount of P was presumably not sufficient to reduce the Cu concentration in the soil solution (Table 2).

The combination of AMF with the natural content of P reduced the Cu content in the shoots (Figure 4), resulting in improved growth of the whole plant (Figure 3). This result could be related to a decreased Cu absorption due to the retention and immobilization of the metal in the cellular wall components of the intra and extraradical hyphae or compartmentalization of the metal in the interior of the fungal cells (Khan et al., 2000; Zhu et al., 2001; Aguilera et al., 2011; Meier et al., 2011, 2012a; Cornejo et al., 2013), reducing Cu transfer to the AM colonized plant. The results obtained by Cabral et al. (2010) demonstrated the myceliuns of *R. clarus* has the capacity to retain higher quantities of Cu compared to Zn, Cd or Pb, corroborating that this AMF can excludes this element outside the root cell and limits plant exposure to it. Additionally, glycoproteins produced by AMF can effectively retain heavy metal in high amounts in the soil, therefore reducing the availability of the contaminants to the plants, with a subsequent plant protective effect. In this sense, glomalin (operationally studied as GRSP) has been widely studied for its implications in sequestering Cu, Cd, Pb, Zn, Cr, and Al (González-Chávez et al., 2004; Chern et al., 2007; Cornejo et al., 2008; Vodnik et al., 2008; Aguilera et al., 2011; Gil-Cardeza et al., 2014; Seguel et al., 2015), showing that this sequestration can be important in the stabilization of contaminated soils. Moreover, Bedini et al. (2009) demonstrated that the amount of Cu, Ni, Pb, and Co bound to GRSP was 2, 3, 0.83, and 0.24 % of the total content of such metals in contaminated soils, respectively. This shows a reducing of both availability of PTE and plant stress, especially due to the presence of these elements in the soil. In the present study, it was demonstrated that inoculation

with *R. clarus* provided increments of GRSP, especially in soil with high Cu contents, which can support more evidence about the beneficial role of AMF on the growth of *C. juncea*, as previously observed by Ferreira et al. (2015).

Another mechanism related to the reduction of PTE uptake is associated with the P nutritional status of the plants. It has been observed that the increase in P supply provides greater retention of Cu in the roots of *C. juncea* and it promotes a reduction in translocation of this element to the shoots. In fact, other studies have demonstrated the formation of insoluble metal-phosphates complexes in plant roots (van Steveninck et al., 1994; Brown et al., 1995). Since the improvement of nutritional status of P can reduce the phytotoxicity of metals, plants may increase the APses excretion into the soil as a mechanism of attenuation in order to prevent high levels of free metal ions in sensitive cellular compartments, as the cytoplasm (Barceló and Poschenrieder, 1992). Thus, well-nourished plants could store P-metal complexes in vacuoles (Barceló and Poschenrieder, 1992) or they can form polyphosphate granules inside the roots (Barceló and Poschenrieder, 1992), in both cases limiting the presence of free metal ions.

In addition, the prevention of absorption by the precipitation or chelation of PTE in the rhizosphere (Göhre and Paszkowski, 2006; Vodnik et al., 2008) could also decrease the transportation of Cu from the roots to the shoots, especially in the soil with the natural content of P. The lower Cu transport to the shoots in the treatments with the natural content of P can also be related to greater glomalin production (GRSPs) in treatments inoculated with AMF (Figure 2). Then, even without an increase in the P inputs, Cu immobilization can be indirectly produced by means of the participation of complementary mechanisms that generate a solubilization of diverse P sources accumulated in the soil as a product of annual hard fertilization. Finally, in the case here studied, the participation of APases and AMF are two well-known systems that increase plant P nutrition but can be combined with other systems as the use of bacteria and free-living fungi with the capability to solubilize organic and inorganic P sources in the soil. These, altogether, allow for a more sustainable use of the scarce P sources, in this case oriented to the bioremediation of Cu-contaminated soils.

CONCLUSIONS

Combination of phosphorus supply and AMF (*Rhizophagus clarus*) inoculation can be an adequate strategy to reduce Cu phytotoxicity in *Crotalaria juncea* as it increases the plant biomass and modify the APase enzyme activity in the soil and plant. Additionally, glomalin produced by the AMF and accumulated in the soil can decrease the availability of Cu to the plants by means of sequestration beyond the root surface, with a consequent plant protective effect. This strategies result in a beneficial interaction that can be considered the basis for the implementation of bioremediation processes in Cu contaminated soils, especially those affected by the use of Cu-based agrochemicals.

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