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Research article

Effects of *Rhizophagus clarus* and P availability in the tolerance and physiological response of *Mucuna cinereum* to copper

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) improve plant ability to uptake P and tolerate heavy metals. This study aimed to evaluate the effect of available P and the inoculation of *Rhizophagus clarus* in a Cu-contaminated soil (*i*) on the activity of acid phosphatases (soil and plant), the presence of glomalin, and (*ii*) in the biochemical and physiological status of *Mucuna cinereum*. A Typic Hapludalf soil artificially contaminated by adding 60 mg kg^{-1} Cu was used in a 3 × 2 factorial design with three replicates. Treatments consisted of three P levels: 0, 40, and 100 mg kg^{-1} P. Each P treatment level was inoculated (+AMF)/non-inoculated (−AMF) with 200 spores of *R. clarus* per pot, and plants grown for 45 days. The addition of at least 40 mg kg^{-1} P and the inoculation of plants with *R. clarus* proved to be efficient to reduce Cu phytotoxicity and increase dry matter yield. Mycorrhization and phosphate fertilization reduced the activity of enzymes regulating oxidative stress (SOD and POD), and altered the chlorophyll *a* fluorescence parameters, due to the lower stress caused by available Cu. These results suggest a synergism between the application of P and the inoculation with *R. clarus*, favoring the growth of *M. cinereum* in a Cu-contaminated soil. This study shows that AMF inoculation represents an interesting alternative to P fertilization to improve plant development when exposed to excess Cu.

1. Introduction

In vineyards, Cu-based fungicide are often applied to prevent foliar fungal diseases. This common practice has led to an increased copper (Cu) content in vineyard soils over the years in most of the traditional wine regions of the world (Rusjan et al., 2007; Herrero-Hernández et al., 2011; Ruyters et al., 2013; Duplay et al., 2014; Babcsányi et al., 2016). It is also the case of Southern Brazil, where sandy soils with low organic matter increase Cu bioavailability (Andreazza et al., 2010; Brunetto et al., 2014; Tiecher et al., 2016a,b). In those soils, contents of pseudototal Cu as high as 62 mg kg^{-1} (USEPA Method 3050B) has been found in the 0–20 cm soil layer, whereas natural contents are close to 3.0 mg kg^{-1} Cu (Miotto et al., 2014). According to the Brazilian environmental agency (CONAMA 420, 2009), soils with Cu content above 60 mg kg^{-1} need preventive or corrective practices to ensure the maintenance of soil functionality or to restore soil quality.

After the eradication of vineyards, generally following decreases in productivity, soils are tilled for the application of limestone and fertilizers. This practice stimulates the mineralization of the organic matter and, consequently, the availability of Cu, since a great portion of the heavy metals is complexed in the organic matter (Brunetto et al., 2014). Soon afterwards, annual soil cover species, such as mucuna (*Mucuna cinereum*), are planted to protect the soil surface from the impact of rainfall, to reduce water erosion, and to favor nutrient cycling (Mackie et al., 2012). However, high Cu in the soil may cause toxicity to cover crops, which can be expressed by the reduction in biomass and consequent reduction of soil cover (Tiecher et al., 2016a,b,c). Excess Cu in the soil causes decrease in the length of roots and number of lateral

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roots, damages in the cuticle, and cracks in important plant organs (Sheldon and Menzies, 2005; Michaud et al., 2008). Furthermore, excessive levels of Cu in plant tissues alter the water content and the osmotic potential. As a result, photosynthesis can be reduced and nutritional imbalance seen (Yruela, 2005). Cu also binds to protein active sites due to its high affinity for sulfhydryl groups, impairing protein function (Ruttkay-Nedecky et al., 2013). Copper toxicity also generates reactive oxygen species (ROS), causing lipid peroxidation, affecting membrane permeability (Andrade et al., 2010). ROS also reduces the photosynthetic rate due to damages in the photosystem II (PSII) (Mateos-Naranjo et al., 2013).

The toxic effect of Cu on plants can be reduced by adopting strategies that decrease soil Cu bioavailability. For instance, the application of P-related fertilizers can reduce Cu toxicity to plants by forming precipitates with Cu in the soil solution (Cao et al., 2003; Kede et al., 2008), or by forming oxide-phosphate-heavy metal ternary complexes with mineral colloids (McBride, 1994; Pérez-Novo et al., 2009). In addition to improving the nutritional status of plants, phosphate may increase the retention of heavy metals (notably Cu) in the roots by forming insoluble metal compounds, therefore reducing the translocation of metals to the shoots (Van Steveninck et al., 1994; Brown et al., 1995), with visible benefits for the plant.

Inoculation of plants with arbuscular mycorrhizal fungi (AMF) is another good strategy to alleviate Cu phytotoxicity. AMF promote immobilization of heavy metals (Gonzalez-Chavez et al., 2004a; Cornejo et al., 2013), increase P-uptake and growth, by diluting the contents of Cu in the tissues of plants (Ferreira et al., 2015). AMF also decrease the uptake through precipitation or chelation of Cu in the rhizosphere (Kaldorf et al., 1999), as well as by the exudation of glycoproteins called glomalins (González-Chávez et al., 2004b). The strategies mentioned above decrease the translocation of Cu from the roots to the shoots, alleviating metal phytotoxicity (Christie et al., 2004).

Based on the ameliorating effects stated above, we expect that the application of P and the inoculation of AMF may alleviate the toxicity of Cu and improve the growth of *M. cinereum* plants introduced in soils with high contents of Cu. Therefore, this study aimed at: (*i*) evaluate the effect of P application and *Rhizophagus clarus* inoculation on the activity of acid phosphatases (soil and plant) and the presence of glomalin, and (*ii*) evaluate the interaction between P and *R. clarus* on some biochemical and physiological changes observed in *M. cinereum* grown in a soil with high Cu-content.

2. Material and methods

2.1. Soil preparation and experimental design

The soil used was a Typic Sandy Hapludalf soil from a natural grassland area from the Campanha Gaúcha, Southern Brazil (29°43′07.04″S; 53°42′29.60″O). Before the experiment was set up, lime was applied to increase soil pH (up to 6.0) and allowed to stabilize for 45 days. Then, the soil was contaminated with the addition of 60 mg kg^{-1} of Cu ($CuSO_4$ $2H_2O$) and let stand for an extra 45 days. Following that period, the soil was autoclaved twice for two hours at 120 °C. Nutrients were added at rates of 100, 30, 5, and 0.80 mg kg^{-1} of N (NH_4Cl), K (K_2SO_4), Zn ($ZnSO_4.7H_2O$), and B (H_3BO_3), respectively. The Nitrogen fertilization was divided into two applications at 15 and 30 days after plant germination. Selected chemical and physical properties after soil preparation are presented in Table 1 (data from representative composite samples). For the study, a completely randomized 3 × 2 factorial design with three replicates was established with three levels of P (0, 40 and 100 mg kg^{-1}), inoculated (+AMF) or non-inoculated (−AMF) with spores of *R. clarus*. Triple superphosphate was used as source of P and the experiment conducted in a greenhouse.

Table 1
Soil chemical and physical characteristics following the application of P and Cu (Ferreira et al., 2015).

Soil characteristics	0 mg kg^{-1} P	40 mg kg^{-1} P	100 mg kg^{-1} P
pH (H_2O)	5.9	5.6	5.5
TOC (g kg^{-1})	6.5	6.5	6.5
Available Cu by EDTA (mg kg^{-1})	45.6	45.5	42.5
Cu^{2+} in soil solution (mg L^{-1})	10.5	7.3	4.8
Available P by Mehlich-1 (mg kg^{-1})	5.6	34.1	85.3
PO_4^{3-} in soil solution (mg L^{-1})	5.2	7.4	10.1
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
Clay (g kg^{-1})	54	54	54
Sand (g kg^{-1})	894	894	894
Silt (g kg^{-1})	52	52	52

2.2. Plant component analyses

Seeds of *Mucuna cinereum* were scarified with concentrated H_2SO_4 for 5 min and then rinsed in distilled autoclaved water. Four seeds were sown in each 3.5 L pot. Ten days later only two plantlets were kept in each pot. The AMF inoculation was performed using 200 spores (multiplied in soil cultivated with *Brachiaria decumbens*) of *R. clarus* per pot.

Forty-five days after emergency (DAE), shoots were cut near the soil surface. A leaf sample was immediately placed in liquid N_2 and stored in an ultrafreezer at −80 °C for biochemical analyses. The dry matter yield (dry matter of shoots + roots) was estimated after the biomass was dried in a forced-air oven (± 65 °C) until reaching constant weight. Root and shoot dry matter was measured with a precision scale. P and Cu concentration in the shoots were determined by ICP-OES (Perkin-Elmer Optima 7000 DV) after HNO_3–$HClO_4$ digestion.

The ability of plants to absorb and translocate Cu was measured by means of a bioaccumulation factor (BF) and a translocation index (TI). BF is defined as the concentration of the element in the plant in relation to the concentration in the soil solution (Malik et al., 2010). On the other hand, TI is obtained by the content of the element in the shoots in relation to the content in the roots of the plants (Cui et al., 2007). The following equations were used for calculations:

$$BF = [Metal]_{shoots} / [Metal]_{soil\ solution} \quad (1)$$

$$TI = [Metal]_{shoots} / [Metal]_{roots} \quad (2)$$

2.3. Mycorrhizal colonization and glomalin determination

Roots were stored in a solution with formaldehyde (40%), alcohol (50%), and glacial acetic acid in the proportion of 13:200:5, followed by a clarification and staining procedure using the Phillips and Hayman method (1970). AMF colonization rate was evaluated on a grid plate following Giovannetti and Mosse (1980). The number of AMF spores was determined in 50 mL of soil by the wet sieving and centrifugation in a sucrose solution method (Gerdemann and Nicolson, 1963).

Easily extractable glomalin (EE-GRSP) and total glomalin (T-GRSP) were estimated by Bradford (1976), modified by 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. Total glomalin (T-GRSP) was extracted with 50 mmol L^{-1} of sodium citrate at pH 7.0 after three 1-h autoclaving cycles at 121 °C. The extractor was separated from the soil through centrifugation at 3500 rpm for 10 min. The protein in the supernatant was quantified by Bradford, using bovine serum albumin (BSA) as standard (Bradford, 1976). The glomalin