ARTIGO ORIGINAL

Interactions of copper and phosphorus in accessions of *Pfaffia glomerata*: effect on growth and yield of β-ecdysone

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ABSTRACT: The increase in copper content (Cu) in soil by antropogenic actions can cause severe damage to plants. This study aimed to evaluate the effects of increasing levels of Cu and phosphorus (P) on the growth and production of β-ecdysone of two accessions (JB e BRA) of *Pfaffia glomerata*. The experiment was carried out under greenhouse conditions in pots containing 4.0 kg of an Ultisol soil with four Cu levels (control, 20, 40 and 80 mg/kg) and three P levels (10, 60 and 120 mg/kg) for 90 days. Root and leaf Cu concentration in JB accession linearly decreased with increasing Cu levels in the presence of 60 mg P/kg soil. On the other hand, in general, at 10 and 120 mg P/kg soil, root and leaf Cu concentration increased with increasing Cu levels. The increase in soil Cu availability reduced the P concentration in leaves. In the presence of 60 mg P/kg soil, total dry matter in JB accession linearly increased with increasing Cu levels. The maximum total dry mass in BRA accession occurred at 40 mg Cu/kg and 10 mg P/kg of soil. The addition of P and Cu only influenced the root β-ecdysone accumulation in JB accession, where at 60 mg P/kg soil it linearly increased with increasing Cu levels. Therefore, both *P. glomerata* accessions showed significant variability for plant biomass production and root β-ecdysone accumulation in relation to changes in soil Cu and P levels, but JB accession was more responsive.
Key words: Brazilian ginseng, heavy metal, P and Cu interaction, secondary metabolite.

RESUMO: Interações de cobre e fósforo em acessos de *Pfaffia glomerata*: efeito no crescimento e rendimento de β-ecdisona. O aumento do teor de cobre (Cu) no solo por ações antrópicas pode causar severos danos aos vegetais. Este estudo teve como objetivo avaliar os efeitos de níveis crescentes de Cu e fósforo (P) no crescimento e produção de β-ecdisona de dois acessos (JB e BRA) de *Pfaffia glomerata*. O experimento foi conduzido em casa de vegetação e em vasos contendo 4,0 kg de Argissolo Vermelho distrófico típico acrescido de quatro níveis de Cu (controle, 20, 40 e 80 mg/kg) e três níveis de P (10, 60 e 120 mg/kg) durante 90 dias. A concentração de Cu nas raízes e folhas no acesso JB linearmente decresceu com o aumento dos níveis de Cu na presença de 60 mg P/kg solo. Por outro lado, em geral, com 10 e 120 mg P/kg solo, a concentração de Cu nas raízes e folhas aumentou com o aumento dos níveis de Cu. O aumento da disponibilidade de Cu no solo reduziu a concentração de P nas folhas. Na presença de 60 mg P/kg solo, a massa seca total da planta no acesso JB aumentou linearmente com o aumento dos níveis de Cu. A máxima produção de massa seca no acesso BRA ocorreu em 40 mg Cu/kg e 10 mg P/kg solo. A adição de P e Cu somente influenciou a acumulação de β-ecdisona nas raízes do acesso JB, onde na presença de 60 mg P/kg solo houve aumento linear com o aumento dos níveis de Cu. Portanto, ambos os acessos de *P. glomerata* mostraram variabilidade significativa para a produção de biomassa e acumulação de β-ecdisona nas raízes em relação aos níveis de Cu e P no solo, mas o acesso JB foi mais responsivo.
Palavras-chave: Ginseng brasileiro, metal pesado, interação P e Cu, metabólito secundário.

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INTRODUCTION

Pfaffia glomerata (Spreng.) Pedersen, known as Brazilian Ginseng, has been used in pharmacology mainly due to its β-ecdysone accumulation in roots (Festucci-Buselli et al. 2008; Flores et al. 2010; Serra et al. 2012). β-Ecdysone is a phytoecdysteroid produced by various plants whose purpose is presumably to disrupt the development and reproduction of insect pests (Thummel and Chory 2002). In addition, some studies suggest that *P. glomerata* has moderate tolerance to toxic elements such as Al, Hg, Cd, Pb and As (Skrebsky et al. 2008; Calgaroto et al. 2011; Gupta et al. 2013; Maldaner et al. 2015). This species has high genetic diversity in natural populations (Kamada et al. 2009), resulting in significant variability, which can promote different levels of tolerance to heavy metals.

The content of secondary metabolites in plants can be altered by heavy metal toxicity (Shützendübel and Polle 2002). However, only a few Brazilian medicinal plant species have been the subject of studies that relate the effects of metals on the secondary metabolites production. Recently, Gupta et al. (2013) reported the effect of Hg, As and Pb toxicity on the phytochelatins (PQ) production of *P. glomerata* plants. The PQ production is considered essential for the detoxification of metal and metalloid ions in plants. However, these authors found that only the addition of As was capable of inducing the production of PQ2, PQ3 and PQ4 in the roots of *P. glomerata*.

The paradox between the essentiality and the potential toxicity of micronutrients has been the subject of recent studies (Yruela 2005; Lequeux et al. 2010). In this context, elements present in pesticides such as Cu have received increasing attention, especially in agricultural areas with a long history of application (Farias et al. 2013; Miotto et al. 2014). Both excess and deficiency of nutrients affect many primary metabolic processes in plants (Marschner 2012).

Farias et al. (2013) observed that tissue P and Cu concentrations in potato genotypes showed a strong correlation with high Cu concentrations in Cambisols, whereas Fe and K tissue concentrations were more strongly correlated with Cu levels in Ultisols. The addition of P enhances the adsorption of Cu (Sarioglu et al. 2005) thus reducing its availability in the soil solution (Liu et al. 2007). Recently, Ferreira et al. (2015) observed that a synergistic effect between P application and inoculation with *Rhizophagus clarus* (arbuscular mycorrhizal fungi) could be of technological interest for achieving increased growth of *Crotalaria juncea* L. plants cultivated in soils with high Cu levels via the promotion of effective mechanisms for reducing Cu phytotoxicity. Based on this, the aim of the present work was to evaluate the effects of increasing levels of Cu and P on the growth and production of β-ecdysone in two accessions (JB and BRA) of *P. glomerata*.

MATERIALS AND METHODS

Two accessions (BRA and JB-UFSM) of *P. glomerata* were multiplied *in vitro* and acclimatized *ex vitro* according to the method of Skrebsky et al. (2008) for this study. An Ultisol soil with a sandy texture was collected (0-20 cm depth) from a natural grassland area (29°43'07.04" S; 53°42'29.60" W). After collection, the soils were dried, ground and reserved for the cultivation of P. glomerata.

A 4 x 3 factorial design was employed in a completely randomized scheme with 6 replicates. The treatments consisted of four levels of Cu (control, 20, 40 and 80 mg/kg soil) and three levels of P (10, 60, and 120 mg P/kg soil). The control level of Cu consisted of the natural content in the soil (3.2 mg/kg) and the other levels were done by addition of $CuSO_4.5H_2O$. Phosphorus was applied as triple superphosphate. A basal fertilization of 40 and 60 mg/kg of K (K_2SO_4) and N (NH_4NO_3), respectively, was applied to all plots. Nitrogen was added after 30 days of transplantation. With the exception of the nitrogen fertilization all other elements were added before 45 days of planting. During this period the soil was incubated in a greenhouse with an 80% maximum water holding capacity (MWHC). The soil data of chemical analysis before the cultivation are shown in Table 1.

The experimental unit was a pot, internally lined with a plastic bag to prevent loss of water and nutrients by drainage, containing 4.0 kg of dry soil (0.5 cm mesh) and one plant. The pots underwent periodic rotation to avoid the location of the greenhouse effect. The reposition of water evapotranspired was performed daily by irrigation with distilled water, up to 80% MWHC by weighing the pot.

Three months after planting, the plants were collected and separated into leaves and roots. Plants were washed gently with distilled water, followed by drying in an oven either at 45 °C with air circulation system to reach constant weight to determine the β-ecdysone content or at 60 °C for the dry matter analysis. The determination of the β-ecdysone content was conducted according to the methodology described by Flores et al. (2010).

All the data were tested by the assumptions of the mathematical model (normality and homogeneity of variance). The analysis of variance of the experimental data was performed using the F-test. The means of the qualitative factors, when significant, were compared by Tukey's test (P <

TABLE 1. Chemical properties of an Ultisol soil supplied with increasing concentrations of Cu and P (mg/kg) before planting of *P. glomerata*. Santa Maria, Brazil.

	Treatments of Cu (mg/kg)/P (mg/kg)											
	0/10	20/10	40/10	80/10	0/60	20/60	40/60	80/60	0/120	20/120	40/120	80/120
Cu (mg/kg)(1)	3.6	21.8	42.7	82.4	2.89	21.9	41.2	83.5	3.1	23.8	42.2	87.1
P (mg/kg)(2)	7.1	5.9	6.7	6.7	59.0	58.4	60.0	57.5	111	117	114	115
K (mg/kg)(2)	78.2	78.6	81.8	83.4	74.0	73.0	46.2	76.2	72.8	80.6	74.2	74.0
Ca ($cmol_c$/dm)(3)	3.9	4.2	3.7	3.9	3.7	3.2	3.0	3.0	3.2	3.1	3.0	3.2
Mg ($cmol_c$/dm)(3)	2.1	2.0	2.3	2..3	2.1	2.1	2.1	2.1	2.1	2.7	2.1	2.3
Al ($cmol_c$/dm)(3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% MO(4)	1.5	1.4	1.3	1.3	1.6	1.4	1.5	1.3	1.5	1.4	1.4	1.4
pH-H_2O(5)	5.7	5.5	5.6	5.6	5.7	5.7	5.6	5.6	5.7	5.6	5.7	5.7

(1) Available Cu in the soil were extracted with EDTA solution (0.01mol/l Ethylenediamine tetraacetic acid, 1 mol/l ammonium acetate, pH 7.0); (2) Available P and K were extracted by filling 5 g of dry soil and 50 ml of Mehlich-1 (HCl 0.05N + H_2SO_4 0.025N); (3) Available Ca and Mg, and Al in the soil were extracted with 1 mol/l KCl; (4) oxidation in sulfochromic solution by heating and determination by spectrophotometry with Cr^{+3}; (5) ratio 1:1.

0.05). The quantitative factor, when significant (P < 0.05), was subjected to polynomial regression analysis, by testing the linear and quadratic models.

RESULTS AND DISCUSSION

Plant growth

Leaf, root and plant dry matter of both accessions were affected upon addition of P and Cu levels, but JB accession was more responsive (Figure 1). The addition of Cu caused a quadratic response in the leaf biomass of both accessions, in which for the BRA accession it was only affected at 60 mg P/kg soil (Figure 1a), whereas in JB the effect of Cu was observed at 10 mg P/kg soil (Figure 1b). In general, the highest dose of Cu (80 mg/kg) caused leaf necrosis (data not shown). The addition of P and Cu did not affect root biomass in BRA accession (Figure 1c). On the other hand, at 10 and 120 mg P/kg soil the root biomass in JB accession showed a quadratic response with increasing Cu levels, which decreased at 80 mg Cu/kg soil (Figure 1d), and at 60 mg P/kg soil it showed a positive linear response (Figure 1d). Total plant biomass in BRA accession was only affected at 10 mg P/kg soil, in which it showed a quadratic response with increasing Cu levels, and the dose of minimum yield was at 80 mg Cu/kg soil (Figure 1e). On the other hand, the addition of P and Cu had a higher effect on plant biomass of JB accession (Figure 1f). While at 10 and 120 mg P/kg soil the plant biomass showed a quadratic response with increasing Cu levels, in which it decreased at 80 mg Cu/kg soil (Figure 1f), at 60 mg P/kg soil it showed a positive linear response (Figure 1f).

Although Cu has important roles in various physiological processes in plants (Yruela 2005), at elevated concentrations it can damage chromatin and the photosynthetic apparatus (Maksymiec 1998), which cause reduction of plant growth (Michaud et al. 2008; Bravin et al. 2010; Farias et al. 2013). The paradox among the essentiality of mineral elements and its potential toxicity to plants generally is well reported for micronutrients (Marschner 2012). On the opposite, the range of macronutrient concentrations considered adequate in plant tissues and soils is very variable (Marschner 2012). In the present study, the addition of 60 mg P/kg soil reduced the toxicity of Cu in plants of JB accession, but at 120 mg P/kg soil there was a decrease for most of the growth parameters assessed, which were below the control treatment (10 mg/kg). The reduction of Cu toxicity effects in *P. glomerata* plants upon addition of P in the soil may be due to the increased soil sorption capacity for Cu. Bolan et al. (1999) found that most Cd sorption was higher in the presence of $H_2PO_4^-$ than by the addition of Cl^-, NO_3^- and SO_4^{2-}. Moreover, simulations using the program MINTEQA2 showed that the P addition in soil increased the sorbed species formation of Cu-P in solution (Pérez-Novo et al. 2009). The optimum levels of P for plant growth vary widely among species and genotypes within a species, and overdosed P may cause toxicity

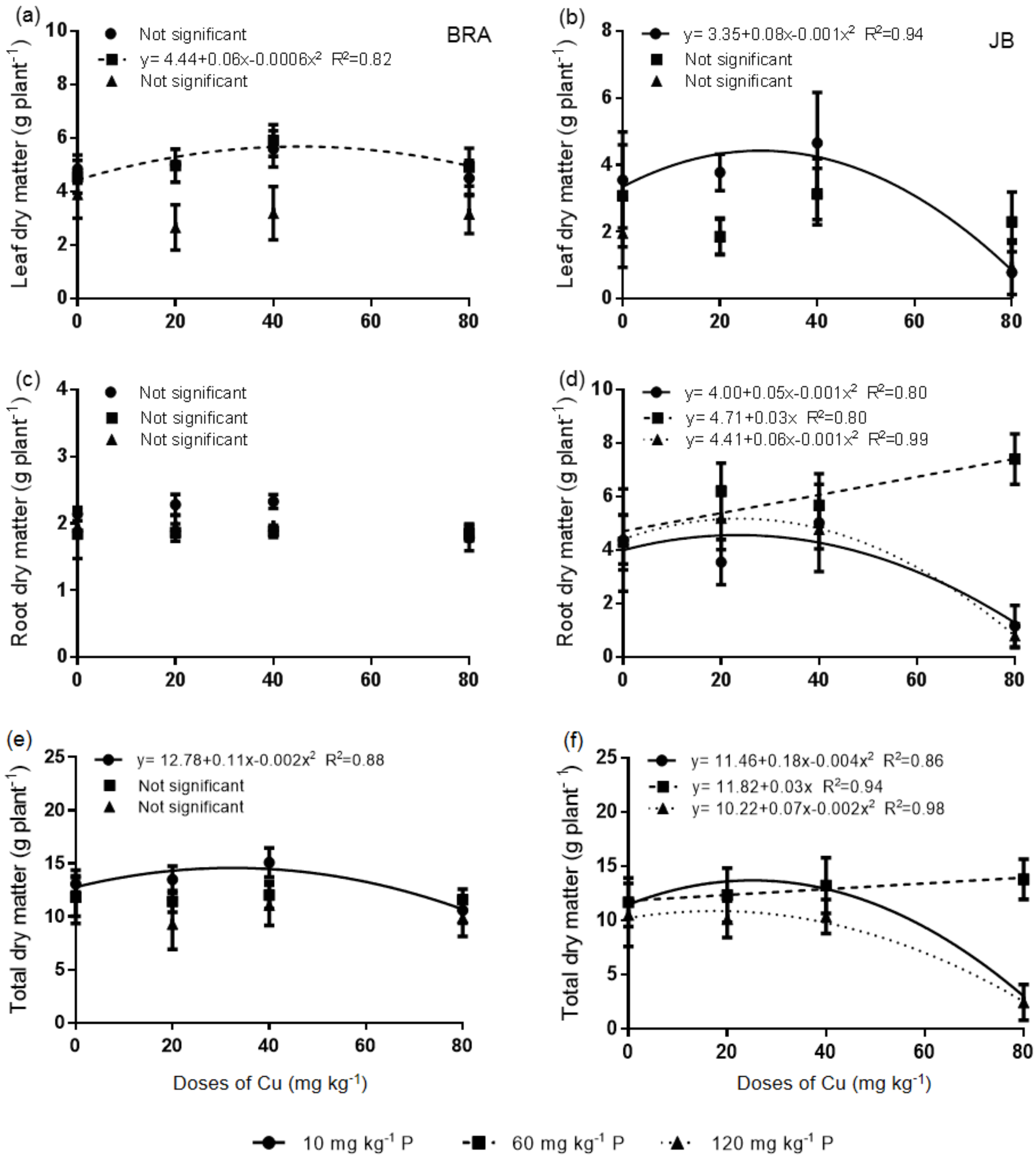

FIGURE 1. Dry matter yield of leaves, roots and plants of two accessions (JB and BRA) of *Pfaffia glomerata* grown in an Ultisol soil supplied with increasing concentrations of Cu and P (mg/kg), Santa Maria, Brazil.

which may be associated with deficiency of other nutrients, particularly of micronutrients such as Zn (Marschner 2012).

The higher root dry matter production of JB than BRA accession at lower availability of P (Figure 1d) is in accordance with other studies and attributed to the adjustment of plant growth under nutrient deficiency, as well as due to the higher genetic diversity for this species in natural populations (Kamada et al. 2009). Skrebsky et al. (2008) reported that plants of *P. glomerata* showed an increase in photoassimilate partition to the roots under low availability of P in the soil. Singh et al. (2013) observed that the fraction of leaf, stem and fruit biomass of *Gossypium hirsutum* L. to the total biomass tended to be lower under P-stress treatments (0.05 and 0.01 mM P), whereas fraction of root biomass to the total biomass increased up to threefold consistently across experiments.

Tissue copper and phosphorus concentrations

The accessions of *P. glomerata* markedly differed in partition of the Cu taken up. The JB accession showed the highest Cu concentration in roots as compared to the leaves; with the highest difference under the addition of 40 and 80 mg Cu/kg (Figure 2). There was only positive correlation (r: 0.61; P<0.05) between root Cu concentration and leaf Cu concentration in JB accession. Interestingly, root and leaf Cu concentrations in JB accession linearly decreased with increasing Cu supply in the presence of 60 mg P/kg soil. On the other hand, at 10 and 120 mg P/kg soil, root Cu concentration increased with increasing Cu supply. The higher root Cu concentration in JB accession may be related, partially, to the concentration effect of the nutrient due to the sharp reduction in dry matter production under the presence of 10 and 120 mg P/kg associated with 80 mg Cu/kg (Figure 1d). The biomass growth reduction by an excess of Cu was also observed in *Arabidopsis thaliana* (L.) Heynh. (Lequeux et al. 2010) and *Solanum tuberosum* L. (Farias et al. 2013).

Overall, tissues P concentrations in JB accession were higher as compared to the BRA accession (Figure 2). In general, the increase in soil Cu availability reduced the P concentration in leaves. Phosphorus concentrations in roots and leaves of both *P. glomerata* accessions increased with increasing soil P availability (Figure 2). Cao et al. (2003) found that phosphate added to the soil was effective in transforming soil Cu (to 13%) from the non-residual to the residual phase. Possibly, P might have hampered the Cu translocation from root to shoot, due to the formation of insoluble compounds capable of reducing the Cu transportation (Soares et al. 2006).

Root β-ecdysone content

The concentration (46 to 66 μg/g dry weight) and accumulation (0.03 to 0.38 g/plant) of β-ecdysone in the roots of *P. glomerata* differed among the accessions and treatments of P and Cu (Figure 3). However, there was only significant correlation (r: 0.74; P < 0.01) between concentration and accumulation of β-ecdysone in BRA accession. Root β-ecdysone concentrations in both accessions were affected upon addition of P and Cu levels, but JB accession was more responsive (Figure 3). While at 10 mg P/kg soil the root β-ecdysone concentration in JB accession decreased linearly with increasing Cu levels, at 60 and 120 mg P/kg soil it showed a quadratic response, in which it decreased at 80 mg Cu/kg soil (Figure 3b). The addition of P and Cu only influenced the root β-ecdysone accumulation in JB accession, where at 60 mg P/kg soil it linearly increased upon addition of Cu (Figure 3d). On the other hand, at 10 and 120 mg P/kg soil, root β-ecdysone accumulation showed quadratic response with increasing Cu levels, in which it showed a high decrease at 80 mg/kg (Figure 3d). However, as the JB root biomass production was generally doubled, the accumulation of β-ecdysone was at least 270% higher for this accession. Changes in β-ecdysone concentration are often observed between different accessions of *P. glomerata* (Festucci-Buselli et al. 2008; Flores et al. 2010), which according to Festucci-Buselli et al. (2008) relates mainly to genetic factors. Kamada et al. (2009b) observed higher divergence among individuals of *P. glomerata* from different populations and lower divergence among those from the same population. In addition, individuals from the population collected at the Shore of Ivaí River had higher β-ecdysone content than other three populations.

The reduction of β-ecdysone concentration in JB accession with the addition of 120 mg P/kg soil may be related to the effect of excess P on the biosynthesis of ecdysteroids. Grebenok et al. (1994) observed that the formation of intermediates of P-ecdysone on spinach inhibit the biosynthesis of phytosterols and phytoecdysteroids. These phosphate conjugates appear to act by negative feedback in regulatory pathways of endogenous biosynthesis of ecdysteroids. Alternatively, these phosphate conjugates may be essential for the transport of ecdysteroids within the plant, or as a means to avoiding the detoxification mechanisms caused by insects that feed from these particular species (Grebenok et al. 1994; Devarenne et al. 1995). Soil abiotic factors have not been studied in relation to β-ecdysone biosynthesis in P. glomerata. The present work is the first to demonstrate the effect of levels of Cu availability on the root β-ecdysone concentration of P. glomerata. Despite the essentiality of Cu, excess of this micronutrient has a strong toxic effect both on plants and animals (Maksymiec et al. 1998).

CONCLUSIONS

The two *Pfaffia glomerata* accessions tested showed significant variability for plant biomass production and root β-ecdysone concentration in relation to changes in soil Cu and P levels, but JB accession was more responsive. The addition of 60 mg P/kg soil decreased the adverse effect of Cu excess on plant growth of both accessions. Moreover, root β-ecdysone accumulation in JB accession linearly increased with increasing Cu levels in the presence of 60 mg P/kg soil.

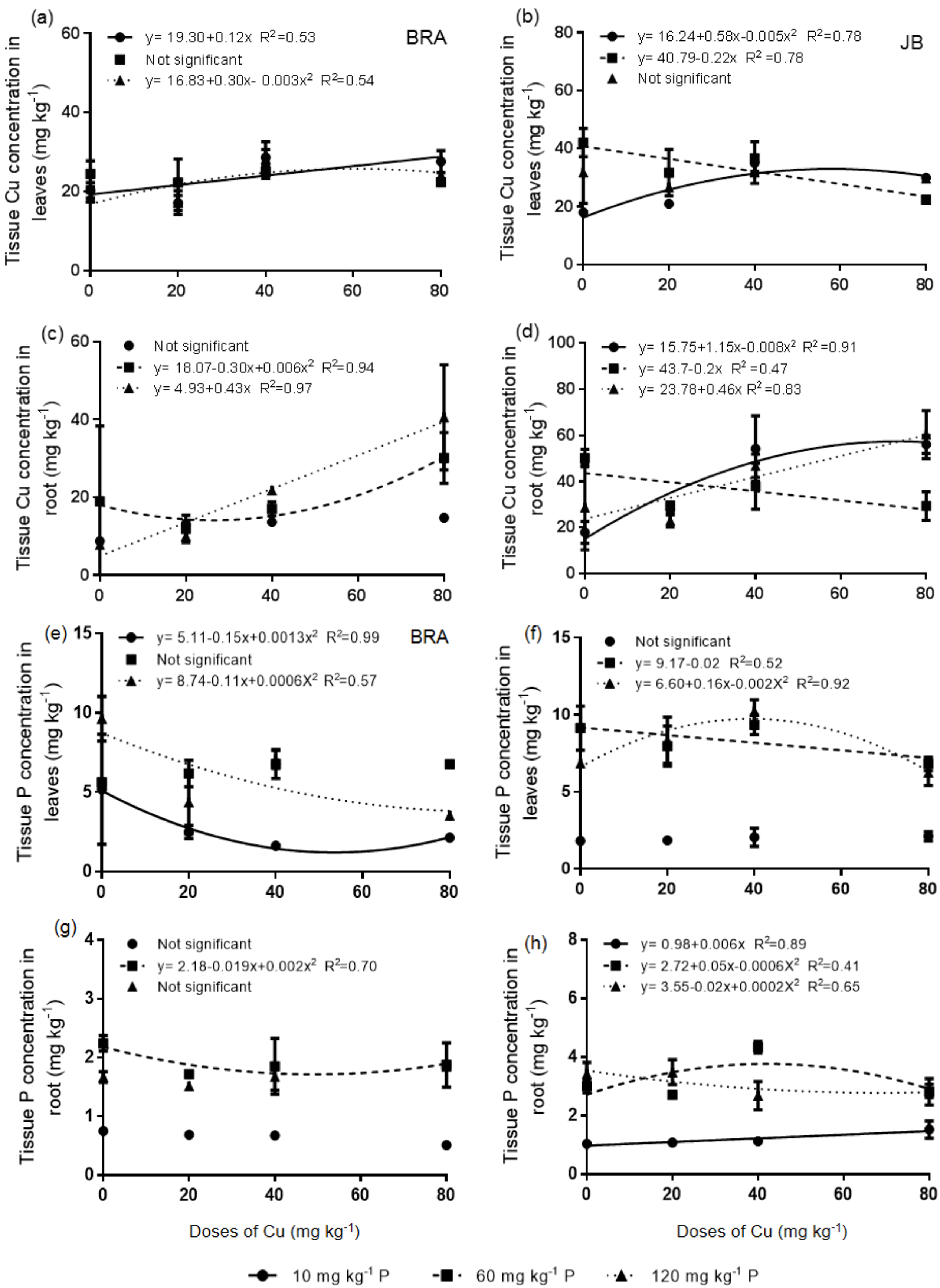

FIGURE 2. Concentrations of Cu (mg/kg dry matter) and P (g/kg dry matter) in dry matter of leaves and roots of *Pfaffia glomerata* plants grown in an Ultisol soil supplied with increasing concentrations of Cu and P (mg/kg), Santa Maria, Brazil.

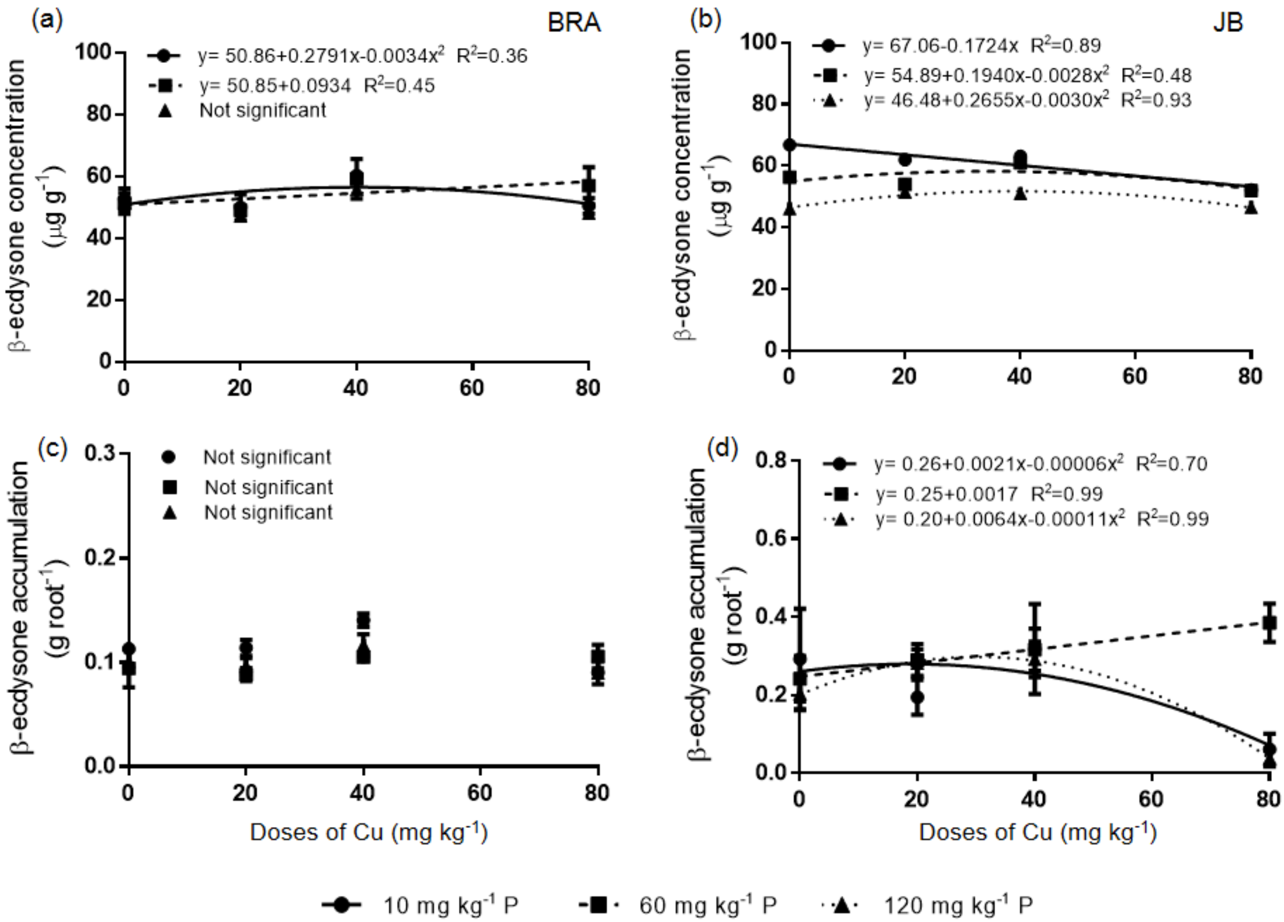

FIGURE 3. Concentration (μg/g dry matter) and accumulation (g/root) of β-ecdysone in roots of two accessions (JB and BRA) of *Pfaffia glomerata* grown in an Ultisol soil supplied with increasing concentrations of Cu and P (mg/kg), Santa Maria, RS.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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