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Structural and ultrastructural variations in roots of *Calopogonium mucunoides* Desv. treated with phenolic compounds from *Urochloa humidicola* (Rendle) Morrone & Zuloaga and phenolic commercial standards

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ABSTRACT

Urochloa humidicola (Rendle) Morrone & Zuloaga is an exotic forage grass cultivated in Brazil. Previous studies have identified different phenolic compounds produced by this species, including flavonoids and *p*-coumaric acid, which have been well-known as allelochemical compounds. We evaluated the morphology, anatomy and ultrastructure of *Calopogonium mucunoides* roots exposed to phenolic compounds found in *U. humidicola*. Leaves of *U. humidicola* were dried, crushed and submitted to extraction in methanol solution, which was fractionated through partition using organic solvents. In the bioassays, the hydromethanolic leaf extract of *U. humidicola*, commercial standard solutions of phenols and a control were all tested against the germination and seedling growth of *C. mucunoides*. The seedlings of *C. mucunoides* were measured, morphologically described, and the roots were processed according to standard techniques for plant anatomy and electron microscopy. *Urochloa humidicola* leaf extract inhibited root development, although they stimulated shoot elongation. The *p*-coumaric acid strongly inhibited plant development, caused root tip necrosis and decreased production of lateral roots, as well as changes in anatomy. Seedlings treated with quercitrin, luteolin and fisetin showed greater lengths, whereas kaempferol negatively affected root growth. Also, *U. humidicola* extract, *p*-coumaric acid and kaempferol caused marked changes in root cell ultrastructure. These results suggest a potential phytotoxic effect caused by *U. humidicola* leaf compounds isolated against *C. mucunoides* seedlings, although the association of these compounds is suggested to be antagonistic.

1. Introduction

Cattle raising in Brazil commonly uses grass-based and extensive grazing systems. Pastures are frequently composed by *Panicum* L. and *Urochloa* P. Beauv. (Poaceae), both providing 90% of animal diet (Bernardi et al., 2012). One of these species is the African-originated *Urochloa humidicola* (Rendle) Morrone & Zuloaga (=*Brachiaria humidicola*), which can grow well on nutrient-poor soils. However, inappropriate pasture management along with a lack of proper nutrient replenishment of the soil system may affect long-term crop productivity (Peron and Evangelista, 2004).

Intercropping grasses and legumes for pastures has been adopted to re-establish nitrogen contents in degraded soils. Forage species of Fabaceae may enrich soil through a symbiosis between their root system and nitrogen-fixing bacteria (e.g. *Rhizobium*). One species that has been adopted for intercropping in pasture systems is *Calopogonium mucunoides* Desv., native legume species of the Brazilian savanna, which can accumulate approximately 300 kg/ha of nitrogen in 12–14 weeks (Seiffert et al., 1985). However, some researchers have reported intercropping inefficiency due to the dominance of grasses, such as *U. humidicola*, over legumes in pastures. Intercropping *C. mucunoides* and *Urochloa decumbens*

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Stapf. and *U. brizantha* (Hochst.) Stapf. has resulted in a decreasing on the dry mass production of the legume species (Euclides et al., 1998).

Secondary metabolites from plants can act on many interactions between plants and their environment. Many of these secondary metabolites, after their release from the donor plant, may exert allelopathy by affecting, positive or negatively, the early development of neighboring plants. Phytotoxicity may be caused by different classes of secondary metabolites, including phenolic compounds (e.g. hydroxycinnamic acids, coumarins, stilbenes and flavonoids) (Reigosa et al., 2013). For example, the allelochemical *p*-coumaric acid, which is a hydroxycinnamic derivative, was found in leaves of *U. humidicola*, and inhibited germination and early development of other plant species (Souza Filho et al., 2005).

Early development may be affected by phenolic compounds, and roots are considered the primary target of many of those micromolecules (Cruz-Ortega et al., 1998). Previous studies have reported phenolic compounds to reduce the size of apical meristematic cells (Cruz-Ortega et al., 1998), to cause necrosis of root tip (Einhellig, 2004) and inhibit cell division and elongation (Chon et al., 2002). Also, phenols may affect cell ultrastructure, and marked structural effects include irregularly shaped plastids and nuclei, and increased cell vacuolation (Cruz-Ortega et al., 1998; Burgos et al., 2004). Furthermore, those phytotoxic compounds may increase generation of reactive oxygen species (ROS) and decrease plant nutrient uptake, photosynthesis and ATP production (Einhellig, 2004; Ding et al., 2007).

It is important to note and describe possible phenolic-induced structural changes on other plant species in order to achieve a better understanding of their phytotoxicity (Ferreira and Áquila, 2000). To date, there has been only a limited number of reports evaluating the effect of isolated phenolic compounds on plant structure and ultrastructure (Chon et al., 2002; Burgos et al., 2004; Ribeiro et al., 2015), and few studies have associated the dominance of *U. humidicola* over legume crops in intercropping systems with morphological and ultrastructural changes caused by phenolic compounds.

Considering earlier reports, that pointed a possible allelopathic effect of *U. humidicola* on legume forage species, and that plant allelochemicals may act either isolated or combined with other compounds, this work aimed to verify if compounds extracted from leaves of *U. humidicola* may inhibit the initial growth of *C. mucunoides*, and compare their joint action with the isolated action of pure phenolic compounds. Also, this study evaluated if these compounds may cause anatomical changes, due to division and differentiation of root tissues, and alter root cell ultrastructure of *C. mucunoides*.

2. Material and methods

2.1. Plant bioassays

To evaluate phytotoxicity of *Urochloa humidicola* compounds, the ethyl acetate fraction of the hydromethanolic leaf extract (EAMLE) was obtained as described by Oliveira et al. (2017). After chromatographic analysis, the following phenolic compounds were identified in the EAMLE extract: *p*-coumaric acid, 3,7,3′,4′-tetrahydroxyflavone (fisetin), 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin), 5,7,3′,4′-tetrahydroxy-3-O-rhamnopyranosylflavone (quercitrin), and 3,7-di-O–α-L-rhamnopyranosylkaempferol (Oliveira et al., 2017).

To evaluate the effects of isolated phenolic compounds, commercial standards of *p*-coumaric acid (PCA), fisetin (FIS), quercitrin (QUE), luteolin (5,7,3′,4′-tetrahydroxyflavone - LUT), and kaempferol (3,5,7,4′-tetrahydroxyflavone - KAE) were acquired from Sigma Chemical Company (St. Louis, USA). These compounds were chosen due to their structural similarity to phenolic compounds from *U. humidicola* EAMLE extracts. Both the isolated phenolic compounds and the EAMLE extract were dissolved in dimethyl sulphoxide (DMSO) (5 $\mu L \cdot mL^{-1}$ of water). For each of the investigated compounds three concentrations were tested: 200, 400 and 800 ppm. Distilled water was adopted as a control. DMSO proportion was kept the same in all treatments, including the control.

Seeds of *C. mucunoides* cv. common (Piraí Sementes, Piracicaba, Brazil) were sterilized in 1% sodium hypochlorite and washed three times with distilled water. For each treatment, triplicates of 10 seeds were placed on two layers of filter paper in plastic Petri dishes (9 cm Ø). The filter papers were moistened with 5 mL of each treatment and the Petri dishes were sealed with Parafilm® (Pechiney, Chicago, USA) and placed in a BOD germination chamber in the dark at 25 °C for 10 days. Total, root and shoot lengths of *C. mucunoides* seedlings were measured at the end of the bioassay (n = 30) using the software Smart Root 4.1 (Lobet et al., 2011) plugin of Image J (NIH, Bethesda, USA).

2.2. Microscopy analysis

Root samples (0.1–1.0 cm from root tip) of *Calopogonium mucunoides* were collected, fixed in 2.5% glutaraldehyde, 4.0% formaldehyde and 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature, and washed in the same buffer. The samples were then dehydrated in an acetone series of 50, 70, 90 and 100%, for 1 h each.

For anatomical analysis, dehydrated root samples were embedded in plastic resin (Historesin®, Leica, Wetzlar, Germany) after dehydration. Cross sections 1–5 μm thick were obtained by rotary microtome (Leica, Wetzlar, Germany), stained with 1.0% toluidine blue O and mounted with Entellan® on glass microscope slides (O'Brien and McCully, 1981). The sections were observed using a brightfield optical microscope (Axioplan, Zeiss, Jena, Germany), and photomicrographs were obtained with a 14 MP camera (Power Shot, Canon, Tokyo, Japan) and Axiovision software (ZEISS, Thornwood, USA).

For ultrastructural analysis, root samples were fixed under the conditions described above, post-fixed in 1.0% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature, washed in the same buffer and dehydrated in an acetone series as described above. The samples were embedded in epoxy resin (Epon®) and polymerized for 48 h at 60 °C. Semi-thin (~0.60 μm) sections were obtained by Reichert Ultracut-S® ultramicrotome (Leica, Wetzlar, Germany) using a diamond knife (Diatome®, Hatfield, USA), and collected in 300 mesh copper grids. The sections were contrasted with 5.0% uranyl acetate for 40 min and lead citrate for 5 min at room temperature (Reynolds, 1963). The samples were observed with a Transmission Electron Microscope (TEM 900, Zeiss, Oberkochen, Germany) at 80 KV.

2.3. Statistical analysis

Images of root cross sections from each treatment, obtained as described above, were used to measure cortex width, and central cylinder and root diameters (n = 3, 25 measurements per root). All measurements obtained were used to determine the Inhibition Percentage, which is given by Equation 1: $\text{inhibition}\ (\%) = [(\overline{X}T - \overline{X}C)/\overline{X}C] \times 100$, where $\overline{X}T$ is the mean of measurements of each treatment, and $\overline{X}C$ is the mean of measurements of the control. Results are expressed as percentages.

Data were statistically analyzed using the R software (R Core Team, Vienna, Austria). Samples normality was evaluated by Shapiro-Wilk test. In order to compare different parameters among treatments, we performed an Analysis of Variance (ANOVA) followed by Tukey test ($p < 0.05$), for parametric data, and the Wilcoxon t test, for non-parametric data.

3. Results

3.1. Seedling morphology

After 10 days, the control seedlings of *C. mucunoides* exhibited white external coloration on the stem and root, and the emission of lateral