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Leaf antioxidant fluctuations and growth responses in saplings of *Caesalpinia echinata* Lam. (brazilwood) under an urban stressing environment

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ABSTRACT

We intended to establish how efficient the leaf antioxidant responses of *C. echinata* are against oxidative environmental conditions observed in an urban environment and their relations to growth and biomass parameters. Plants were grown for 15 months in four sites: Congonhas and Pinheiros, affected by pollutants from vehicular emissions; Ibirapuera, affected by high O_3 concentrations; and a greenhouse with filtered air. Fifteen plants were quarterly removed from each site for analysis of antioxidants, growth and biomass. Plants growing in polluted sites showed alterations in their antioxidants. They were shorter, had thicker stems and produced less leaf biomass than plants maintained under filtered air. The fluctuations in the levels of antioxidants were significantly influenced by combined effects of climatic and pollution variables. The higher were the antioxidant responses and the concentrations of pollutant markers of air contamination in each site the slower were the growth and biomass production.

1. Introduction

Caesalpinia echinata (Caesalpinioideae–Leguminosae) is a native tree species from the Atlantic Rain Forest, occurring along the eastern coastal region of Brazil. It is an endangered species (Cardoso et al., 2005) due to the intense forest fragmentation and to its overexploitation by the Brazilian colonizers for extracting brazilin.

Native forest plantations in urban areas are among the strategies that could facilitate the conservation of biodiversity (Montagnini, 2001; Karnosky et al., 2005). As *C. echinata* presents interesting ornamental features such as the vigorous architecture of adult trees, offering comfortable shade, and the admirable yellow influorescences, it could potentially be planted on a wide scale in urban areas. However, the success of this activity will depend on whether it can tolerate the stress conditions of the urban environment.

Both meteorological conditions and gaseous and particulate pollutants are important stressing factors in large urban centers (Molina and Molina, 2004) and can induce chemical, biochemical, physiological and morphological changes in plants (Bechtold et al., 2005; Ledford and Niyogi, 2005; Iriti et al., 2006). These effects are mainly consequences of the action of reactive oxygen species (ROS) produced more intensively during the exposure of plant to fluctuating environmental factors. Moreover, plants might mobilize antioxidants as protection against excessive or inappropriate ROS production, thus maintaining the oxidative/antioxidative equilibrium and minimizing the stressing consequences, such as disturbances on growth (Foyer and Noctor, 2005; Nali et al. 2005; Sinha et al., 2005; Iriti and Faoro, 2008). Efficient antioxidant system is then an attribute of tolerant plants in urban areas.

Bulbovas et al. (2005) previously verified that air temperature was an important factor for determining seasonality in glutathione levels and in the activity of peroxidases and superoxide dismutase in leaves of potted plants of *C. echinata* growing under both filtered air and meteorological variations naturally observed in the city of São Paulo. Moreover, both ascorbic acid level and peroxidase activity were considerably higher than those found in other Brazilian trees growing under the same environmental conditions (Domingos et al., 1998; Klumpp et al., 1998; Klumpp et al., 2000; Moraes et al., 2002). Based on this information, the following hypothesis was raised: saplings of *C. echinata* would tolerate the oxidative stress imposed by multiple environmental factors in a megacity like São Paulo if the antioxidant fluctuations (total ascorbic acid, thiols and glutathione, superoxide dismutase and peroxidases) closely follow the variations on environmental factors, then minimizing disturbances on plant growth and on

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biomass production. Thus, the present study intended to establish how efficient the leaf antioxidant responses of *C. echinata* are against the varying levels of oxidative factors observed in an urban environment and their relations to growth and biomass parameters and to determine the suitability of *C. echinata* for ornamental purposes in large cities.

2. Materials and methods

2.1. Experimental sites and design

The field experiment was performed in four sites situated in the city of São Paulo, SE Brazil, distinguished by air pollution contamination: Congonhas (23°36'S and 46°39'W) and Pinheiros (23°33'S and 46°42'W), near great avenues, are contaminated by high levels of NO_2, SO_2 and particulate material from vehicular emissions and by low levels of O_3; Ibirapuera Park (23°35'S and 46°39'W), a green area where lower levels of pollutants from vehicular emissions and high O_3 concentrations are registered; a greenhouse (23°38'S and 46°37'W) with low levels of both particulate and gaseous pollutants provided by fiberglass and charcoal filters.

Two-year old saplings of *C. echinata* from an experimental nursery were transferred to plastic pots containing substrate composed by *Pinus* bark. They were cultivated from seeds collected in plant matrices originated from a natural population of *C. echinata*. They were maintained inside the greenhouse under filtered air and were biweekly fertilized. All of them were in average 25 cm height, and presented four composite, bipinnate and alternate leaves inserted in consecutive nodes of the stem, and no branches.

In April 2002, 130 saplings of *C. echinata* were transported to each site. Every 12 weeks, 15 plants were taken from each site for analyses of leaf antioxidants and of growth and biomass parameters. In this manner, samplings were performed in July and October 2002, and in January, April and July 2003, after 3, 6, 9, 12 and 15 months of exposure respectively. During all this period, the plants were maintained under similar condition with respect to nutrition, watering and sun exposure (they were arranged in the field using a compass). The frames supporting the water reservoirs did not have devices for reducing the influence of wind and for shading. In brief, a real condition of plant exposure to the urban environment at the polluted sites was simulated. Nylon wicks guaranteed the water supply during experiment. Plastic boxes served as water reservoirs (Klumpp et al., 1994). Peters (NPK 10:30:20) solution was used for fertilization biweekly

2.2. Air pollution and climate monitoring

Temperature and relative humidity were continuously measured by thermo-hygrographers in all sites. Periodic measurements of photosynthetic photon flux density (PPFD) were made by means of a by Li-Cor Li-185B, aiming at to determine the seasonal profile of this factor in all sites during all the experimental period. The instantaneous measurements were always performed at the same time of the day in each site and were repeated in many days of each trimester. Five instantaneous and consecutive measurements were always performed in the period of each day when maximal exposure to sun light would potentially occur (between 10 am and midday). Rainfall was measured by means of two samplers installed next to the frames in each open site. Atmospheric pollutant concentrations in Congonhas, Pinheiros and Ibirapuera were continuously provided by monitoring stations installed next to the saplings exposure frames. Data on air quality inside the greenhouse were weekly taken. PM_{10} concentrations were estimated gravimetrically by passing high known volumes of air through glass fiber filters for 24 h (ABNT, 1997). SO_2, NO_2 and O_3 were sampled using an aqueous scrubber technique that directed known volumes of air through a filter to remove aerosol particles and then through specific adsorbent solutions (hydrogen peroxide, Griess–Saltzman reagent and potassium iodide, respectively for SO_2, NO_2 and O_3), following titration for SO_2 and spectrophotometric determination at 550 nm (NO_2) and 352 nm (O_3) (ABNT, 1993; Saltzman, 1960).

2.3. Antioxidant analyses

In each sampling month, the leaf antioxidants were analyzed in the totally expanded leaves inserted on the second to fourth youngest nodes of the stem, as previously defined by Bulbovas et al. (2005) based on their antioxidative responses.

Total ascorbic acid was determined in fresh leaves (0.2 g), homogenized with 12 mL of EDTA-Na_2 (0.07%) and oxalic acid (0.5%). The mixture was centrifuged at 40 000*g* and 2 °C for 30 min. An aliquot of the supernatant was added to 2.5 mL of DCPiP (0.02%) and absorbance was measured spectrophotometrically at 520 nm. After the addition of 0.05 mL of ascorbic acid (1%), a second absorbance measurement was performed. Both absorbance measurements were used to estimate the ascorbic acid content (Keller and Schwager, 1977).

The contents of thiols and glutathione were determined according to the method described by De Kok et al. (1985). Fresh leaves (0.7 g) were homogenized with 12 mL of ascorbic acid (0.15%) and centrifuged at 44 000*g* and 2 °C for 30 min. After that, 0.2 mL of DTNB (0.04%) in potassium phosphate buffer (2 M pH 7.0), 0.2 mL of phosphate buffer (0.02 M pH 7.0) and 1.3 mL of tris buffer (pH 8.0) were added to the supernatant. The absorbance was measured spectrophotometrically at 415 nm and the concentration of thiols was determinated. The extracts were then deproteinized by incubating in a water bath at 100 °C for 3 min and further centrifuged at 24 000*g* and 2 °C for 15 min. Absorbance of these extracts was measured in the same manner described before, determining the concentration of deproteinized compounds. Subsequently, glutathione in the deproteinized supernatant was converted to S-lactylglutathione with methylglyoxal (0.09 mL, 0.1 M) and glyoxilase I (0.01 mL), after adding 0.2 mL of DTNB (0.04%) in potassium phosphate buffer (2 M pH 7.0), 0.2 mL of phosphate buffer (0.02 M pH 7.0), 1.1 mL of tris buffer (pH 8.0) and 0.3 mL of albumin (1%). The content of glutathione was calculated by subtracting the concentration of all small molecules remaining after protein is removed and glutathione is converted to S-lactyglutathione from the concentration of non-protein sources following de-proteinization. The proportion of glutathione relative to the concentrations of thiols, expressed as a percentage, was also estimated, considering that this estimation might indirectly indicate a leaf antioxidant response.

Unspecific peroxidases activity was determined in frozen leaves at ~40 °C (0.3 g), homogenized with 12 mL of potassium phosphate buffer (0.1 M, pH 7.0) in the presence of a pinch of polyvinyl polypyrrolidone (PVPP) 2%. The homogenate was centrifuged at 40 000*g* and 2 °C for 30 min. The peroxidases were determined in a reaction mixture of plant extracts, 0.1 M potassium phosphate buffer (pH 5.5) and phenylenediamine (1%), to which was added an aliquot of H_2O_2 (0.3%), according to Klumpp et al. (1989). POD activity was measured spectrophotometrically following the increase in absorbance (ΔE) at 485 nm due to the formation of diaminophenazine (DAP), a product of the oxidation of phenylenediamine (PD) by hydrogen peroxide, catalyzed by peroxidase (Mekler and Bystryak, 1992). Peroxidase activity was defined as a change of the absorbance at two different times in the linear region of the curve.

Superoxide dismutase activity was also determined in frozen leaves at −40 °C (0.4 g), homogenized with 12 mL of potassium phosphate buffer (50 mM pH 7.5) with EDTA-Na_2 1 mM, NaCl 50 mM and ascorbic acid 1 mM in the presence of a pinch of polyvinyl polypyrrolidone (PVPP) 2% and centrifuged at 22 000*g* and 2 °C for 25 min. The activity of SOD was assayed by measuring the SOD inhibition of the NBT photochemical reduction (Osswald et al., 1992). Each reaction mixture contained 0.5 mL of EDTA-Na_2 0.54 mM, 0.8 mL of potassium phosphate buffer (0.1 M, pH 7.0), 0.5 mL of methionine 0.13 mM, 0.5 mL of NBT 0.44 mM, 0.2 mL of riboflavin 1 mM and 0.2 mL of leaf extract. The samples were incubated for 20 min under a fluorescent lamp (80 W). The absorbance of the reaction mixture was determined at 560 nm. A similar mixture lacking the leaf extract was used as control, and a dark control mixture served as blank. The enzymatic activity was expressed as the amount of extract needed to inhibit the reduction of NBT by 50%.

2.4. Growth and biomass measurements

In each sampling month stem height and stem diameter at substrate level were measured in all remaining plants in each site. The dry mass of leaves and of entire plant (leaves, stems and branches, roots) was determined in the same plants sampled for antioxidant analyses. The results of growth and biomass production obtained every 12 weeks were expressed as absolute values.

2.5. Statistical analyses

The quarterly data on antioxidants and on growth and biomass were compared by analyses of variance with two factors (exposure period and site) followed by Tukey test ($p < 0.05$). If necessary, appropriate transformation of data was performed to reach normal distribution and/or equal variances. Adjusted models of leaf antioxidant variations over time in plants from each exposure site were then proposed on the basis of the results of both statistical procedures. For growth and biomass production, significant differences among sites in the same sampling month and among quarterly measurements in the same site were detached by small and capital letters respectively.

Multivariate analyses were performed in two different stages. Firstly it was verified whether the quarterly variations in leaf antioxidants during the exposure time could be predicted by quarterly oscillations in environmental variables. The data were grouped on two matrices taking into account concentration of characteristic air pollutants in the sampling sites. Matrix 1 mainly emphasized the plant exposure to ozone and matrix 2 to pollutants from vehicular emissions (Table 1). The data gathered in each matrix were analyzed by means of the stepwise method, adopting the antioxidant responses as the dependent variables and the abiotic factors as independent variables. The adjustment procedure started from the saturated model (with all the variables present), removing successively those that did not significantly explain the variations in the antioxidants. In the second stage, it was evaluated if the growth and biomass production (dependent variables) during the exposure period could be predicted by variations in leaf