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Climatic factors influence leaf structure and thereby affect the ozone sensitivity of *Ipomoea nil* 'Scarlet O'Hara'

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ABSTRACT

Phenotypic plasticity of the leaves can interfere with the plant sensitivity to ozone (O_3) toxic effect. This study aimed to assess whether the leaf structure of *Ipomoea nil* changes due to climatic variations and whether these changes affect the species' sensitivity. Field exposures, in different seasons (winter and spring) were made. The leaves that developed during the winter were thinner, with a lower proportion of photosynthetic tissues, higher proportion of intercellular spaces and lower density and stomatal index compared to those developed during the spring. The temperature and relative humidity positively influenced the leaf thickness and stomatal index. The visible injuries during winter were positively correlated with the palisade parenchyma thickness and negatively correlated with the percentage of spongy parenchyma; during the spring, the symptoms were positively correlated with the stomatal density. In conclusion, the leaf structure of *I. nil* varied among the seasons, interfering in its sensitivity to O_3.

1. Introduction

The Metropolitan Region of São Paulo city (MRSP) is composed of 39 cities with a population of approximately 20 million habitants representing 75% of the population of São Paulo State. There are 8.5 million vehicles in this area, which contribute to high emission levels of primary pollutants in the air as a result of fuel combustion.

As a result of complex photochemical reactions, ozone (O_3) is formed throughout the year in tropical environments (Moura et al., 2014b). O_3 is considered a powerful phytotoxic pollutant that can cause oxidative stress to vegetation, leading to the development of specific visible injury in the leaves due to reactive oxygen species (ROS), which react with lipids membranes (Schraudner et al., 1998), inducing a type of programmed cell death known as hypersensitive-like reaction (HR-like, Vollenweider et al., 2003; 2013; Moura et al., 2014a).

An extended and integrated assessment of environmental indicators for global change is one of the most important emerging research field (Serengil et al., 2011) and biomonitoring programs are an efficient alternative consistently for monitoring the levels of O_3 once the exposure is made following a standardized method during a time series using specific sensitive species (Klumpp et al., 2001).

The intimate relation between the leaf structure and its physiological role has been discussed for a long time (Toth, 1982; Roderick et al., 1999). Within different populations of the same species, functional leaf traits may vary in relation to the extent that the plant succeeds in acclimating to ambient oxidative pressure (Bussotti, 2008). Higher palisade cell density and the greater spongy mesophyll intercellular space may result in a larger amount of free cell surface able to interact with O_3 (Bennet et al., 1992). Relatively large substomatal chambers and abundant intercellular spaces are anatomical features that reduce the resistance of leaves to gas exchange and therefore favor O_3 diffusion within the leaves (Calatayud et al., 2011).

One of the most interesting endogenous differences that may lead to O_3 exposure sensitivity is stomatal density (Paoletti and Grulke, 2005). Stomatal distribution and behavior respond to a wide range of environmental stimuli such as light, humidity, and CO_2 (Paoletti and Grulke, 2005). Stomatal responses to air pollutants vary among species, leaf and tree age, and in conjunction with other environmental stressors (Mansfield, 1998; Robinson et al., 1998). Differences in stomatal density can also be correlate with differences in the O_3 sensitivity of genotypes of the same species (Ferdinand et al., 2000).

Ipomoea nil 'Scarlet O'Hara'is an O_3-sensitive species (Nouchi and Aoki, 1979) that had been tested in tropical and subtropical environments (Moura et al., 2011; Ferreira et al., 2012). However, the efficiency of *I. nil* 'Scarlet O'Hara' for quantitative O_3

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biomonitoring may be compromised in the subtropics, as the percentage of visible leaf injury may not be directly associated with increased levels of O_3 in the atmosphere (Ferreira et al., 2012). Based on this statement and considering the phenotypic plasticity of this species, we hypothesize that differences in the leaf structure may affect the responsiveness of *I. nil* 'Scarlet O'Hara' to O_3, compromising its bioindicator efficiency.

Considering the hypothesis that plants with differences in their foliar structures may respond in a distinct way to O_3 oxidative stress when used as bioindicators, this study aimed to answer the following questions: (i) Can seasonal climatic variations cause changes in the foliar structure of *Ipomoea nil* 'Scarlet O'Hara'? If so, (ii) what are the climatic variables that influence leaf development and leaf morphology? and (iii) which leaf characteristics are related to the formation of visible injury due to oxidative stress induced by O_3?

2. Material and methods

2.1. Plant cultivation and exposure

The field experiments were conducted in ambient conditions at the Ibirapuera Park in the city of São Paulo, SE Brazil (23°34′55″S and 46°39′25″W), situated 750 m above sea level and next to an air monitoring station of the Environmental Company of São Paulo State (CETESB), where the O_3 levels are recorded along with the climate parameters temperature, humidity and global solar radiation. Ibirapuera Park is located between avenues with heavy vehicular traffic and is historically affected by high and increasing levels of O_3 (Ferreira et al., 2012).

The local climate is subtropical with an average annual temperature of 18.3 °C and with mild winters and summers with high temperatures. The relative humidity is usually above 60%. The average annual rainfall is 1368 mm and is concentrated mainly in the summer (Santos and Funari, 2002).

Seeds of *I. nil* 'Scarlet O'Hara' were germinated in a 10 cm^2 plastic box filled with *Pinus* bark (Plantimax-Eucatex™) and fine vermiculite (3:1) as substrate. After the second leaf emerged, the seedlings were transplanted to pots containing the same substrate. The water supply was guaranteed by capillarity according to the VDI protocol (VDI, 2003). All of the plants received 100 ml Hoagland nutrition solution weekly (Epstein, 1975). The aluminum racks containing the plants were covered with a 50% screen for shading.

Two exposures of 28 days, the first during the winter (from 25/08 to 22/09/2006), and the second during spring (from 14/11 to 11/12/2006) were performed. As a reference, the same number of plants was kept inside a greenhouse (control) with filtered air and controlled temperature.

2.2. Visible injury and structural evaluation

Twenty seven plants were exposed during each season. During the exposure period, sub-lots of three plants were taken at intervals of three or four days (across a total of nine sampling days). The average percentage of the injured leaf area was quantified as described in Ferreira et al. (2012), estimated in classes of 5%. On every sampling day, samples of the 5th, 6th and 7th oldest leaves on the main stem of each plant were selected for structural analysis. From each sampled leaf, six sub-samples of 2 cm^2 were randomly selected according to the methodology proposed by Kubínová (1994). In total, we evaluated 486 samples of 81 leaves of 27 plants during each exposure season.

The samples were fixed under vacuum in a solution of FGAA (Lersten and Curtis, 1988 *apud* Kraus and Arduin, 1997) for 48 h and kept in a 70% ethanol solution. The inclusion was performed in polyethylene glycol 2000 (Richter, 1981; modify), and 5 μm sections were obtained using a rotating microtome (Olympus - Cut 4055). The materials were stain with an aqueous solution (9:1) of astra blue and safranin (1%), and mounted in 66% glycerin.

For the foliar epidermis analysis, the samples were cleared in 5% NaOH, bleached in sodium hypochlorite (50%, v/v), washed with distilled water (Strittmatter, 1973 modify), stained with an aqueous solution (9:1) of astra blue and safranin (1%), dehydrated in an ethanol series and mounted in Permount™ resin.

On leaf blade transverse sections, the thickness of the epidermis cells (adaxial and abaxial), palisade and spongy parenchyma was measured, and the percentage of each leaf tissue including the intercellular space was calculated based on the stereological technique of point counts (Parkhurst, 1982). On paradermal sections, the stomata density and stomata index (SI) (Salisbury, 1927; *apud* Wilkinson, 1979) were calculated, with the latter based on the formula:

$$SI = St \times 100/Sp + St$$

where St is the number of stomata in a determinate area, and Sp is the number of epidemic cells in the same area.

The average of six measurements on each leaf was calculated to provide one data value for each leaf. All of the observations were made using a BX41 microscope (Olympus Optical Company, Tokyo) that was equipped with an image capture system and a semi-automatic system for quantitative measurements, software Pro-Express 4.0.1, Media Cybernetics.

2.3. Statistical analysis

The differences between the 5th, 6th and 7th leaves were tested using a one-way ANOVA considering all of the data. A three-way ANOVA was used to verify the differences between the two exposure seasons (factor 1) along the time series (factor 2) and to compare the plants that were exposed at Ibirapuera Park with those that were kept inside the greenhouse as references (factor 3), using the anatomical measurements as dependent factors. An analysis of variance test was followed by a post-hoc multiple comparison test (Holm-Sidak $p < 0.05$). In every case, the normality was verified by the Shapiro–Wilk test, and the variance around the regression line (homoscedasticity) was tested.

Multiple linear regression analyses were performed to determine whether the measured variables might be predicted using fluctuations in the meteorological variables (air temperature, relative humidity and global solar radiation). All of the data of each sampling day of exposure were jointly analyzed using the backward stepwise method using the microscopic indicators as dependent variables and the average of the environmental parameters between the first day of exposure and each particular day of sampling as independent variables.

A Pearson correlation ($p < 0.05$) was performed to determine which structural variable was related to the development of visible injury.

For all analysis the individual plant was treated as an unit ($n = 27$).

3. Results

3.1. Ozone and climate parameters during the exposures

Regarding the climate parameters (Fig. 1A), the hour average temperature, relative humidity and solar radiation were higher during the spring (21 °C, 81% and 187 Wm^2) compared to during the winter (19 °C, 75% and 173 Wm^2). Inside the greenhouse, the temperature and relative humidity were lower during the winter (23.4 °C and 66%, respectively) compared to during the spring (27 °C and 72%, respectively).

The daily O_3 distribution at Ibirapuera Park was typical of urban areas, where the highest values were registered between 12 and 18 h (Fig. 1B) following the solar radiation distribution pattern (Fig. 1A). During the winter exposure, the daily O_3 distribution presented higher average and pick values when compared to during the spring exposure (Fig. 1B).

3.2. Structural analyses

Considering both of the exposures and time series, only minor differences were detected by the statistical analysis in the structural parameters of 5th, 6th and 7th leaves (data not show) and throughout the exposure period. For this reason, we decided for subsequent analyses to gather data and work with the plant average of each sampling date looking for differences between the exposures.

The plants that were exposed during the winter presented thinner tissues (Fig. 2 and Fig. 3) compared to those of the plants that were exposed during the spring. The percentage of palisade parenchyma cells was higher in the plants from the spring exposure, whereas the percentages of the epidermis and the intercellular space among the palisade parenchyma cells were greater in the plants from the winter exposure (Fig. 4); furthermore, the plants from the spring exposure showed a higher stomatal density and stomatal index on both the adaxial and abaxial epidermis (Fig. 5) compared to the plants that were exposed during the winter.

3.3. Influence of the climate on the leaf structure development and ozone sensitivity

The multivariate regression analysis indicated that the variations in the leaf structure could be mostly explained by significant