RESPONSES TO O_3 - INDUCED STRESS IN DIFFERENT - AGED LEAVES OF SAPLINGS OF A TROPICAL WOODY SPECIES

J.M. Pina

R.M. Moraes

Companhia de Tecnologia e Saneamento Ambiental de São Paulo, 05459 - 900 São Paulo, SP, Brazil; 2. Instituto de Botânica, CP 3005, 01061 - 970 São Paulo, SP, Brazil Tel. 55 11 5073 - 6300 regmorae@uol.com.br

INTRODUCTION

Tropospheric ozone (O_3) is currently the pollutant that causes the greatest injuries to vegetation and losses in crop productivity on a global scale. Its production is the result of a series of photochemical reactions between nitrogen oxides (NOx) and volatile organic compounds (VOC). The city of São Paulo, in the southeast of Brazil, possesses a large and aging automotive fleet and presents favorable climatic conditions for O_3 formation throughout the year. Therefore, O_3 is the atmospheric pollutant that most frequently exceeds the legal standards of air quality in São Paulo (CETESB, 2008).

Inside the leaf, O_3 reacts with cell wall and plasma membrane components, producing reactive oxygen species (ROS). ROS are inherent to plant life because, in equilibrium with the antioxidant system, they participate as second messengers in many physiological processes (Foyer & Noctor, 2005). However, by increasing the production of ROS, O_3 interferes with this equilibrium, causing cellular perturbation that can lead to reductions in photosynthetic rate, premature leaf senescence, leaf necrosis and reduction in productivity and growth (Sanz *et al.*, 002).

The O_3 - induced responses of certain plant species can be used for biomonitoring of air quality. Biomonitoring allows to identify areas experiencing phytotoxic concentrations, to detect chronical pollution levels, and to evaluate the risks imposed to the species in question and to vegetation in general. Nevertheless, species from tropical regions known to act as bioindicators are scarce. Psidium guajava 'Paluma' is sensitive to O_3 and forms typical foliar symptoms (Furlan *et al.*, 007).

OBJECTIVES

This study aims to evaluate additional aspects of the response of Psidium guajava 'Paluma' to O_3 with the objective of establishing its suitability for use as a tropical O_3 - bioindicator species. The evaluated parameters were foliar injury, net photosynthesis, stomatal conductance, activity of the superoxide dismutase enzyme (SOD) and concentration of ascorbic acid (AA). As it is known that O_3 induces the anticipation of leaf senescence, different - aged leaves were analyzed in order to find out whether the young leaves have a greater defense capacity with respect to O_3 - induced stress due to a more efficient antioxidant system. We tested the hypothesis that the carbon assimilation of the older leaves is more reduced as compared to the younger ones resulting in a lower capacity to detoxify active oxygen species in old leaves. As a consequence, the latter show more severe foliar injuries than the former.

MATERIAL AND METHODS

Ibirapuera Park ($23^0$35'S-$46^0$39'W, 690 m asl) experiences the highest ozone concentrations in São Paulo City. The study was carried out in an open field close to an air quality monitoring station operated by the state agency for environmental protection (CETESB), located within the park's facilities. Three O_3 exposures were conducted at this site corresponding to the spring, summer and autumn (03/Oct/2006 to 08/Jan/2007; 08/Jan/2007 to 09/Apr/2007 and 09/Apr/2007 to 22/Jun/2007). During the final period, saplings were exposed to an additional O_3 - polluted site located at the Botanical Institute of São Paulo, were a mobile air quality monitoring station from CETESB was located during this period. In each exposure, 20 saplings of P. guajava 'Paluma' were grown on the high - ozone and on the reference site (greenhouse with filtered air), respectively. Hourly ozone concentrations, air temperature and relative humidity in Ibirapuera were obtained from CETESB (www.cetesb.sp.gov.br). O_3 concentrations at Botanical Institute were continuously with a Horiba monitor. Saplings of P. guajava 'Paluma' approximately 30 cm in height and with 12 leaves were obtained from a nursery. They grow in 1,5 l pots with a mixture of bark from Pinus, vermiculite and coconut fiber (2:1:1) and were fertilized monthly with 100 ml of a solution containing NPK

(20:20:20). Irrigation by capillarity was used. Saplings were examined every day until the onset of foliar injuries, and then at weekly intervals.

Ozone–induced foliar symptoms in P. guajava 'Paluma' were characterized by dark - colored stippling (reddish) between the veins on the upper surface of older leaves, which became more pronounced as the duration of exposure increased, when the injuries began to cover a larger surface of the leaf and reached younger leaves (Furlan *et al.*, 007). The incidence of foliar injury (number of injured plants/total number of plants x 100), severity of injury (number of injured leaves/total number of leaves x 100), and a leaf injury index (a weighted mean) were determined.

Analyses of gas exchange were performed at the end of each exposure periods. Measurements of carbon assimilation under saturating light conditions (Asat), stomatal conductance (gs), transpiration rate (E), and internal CO2 concentration (Ci) were made using an infrared gas analyzer (LCPro+, ADC, UK) with an optional light source. Carbon assimilation under a saturating PAR (1000 μmol photons m - 2s - 1) were measured for six plants from each experimental site, between 9:00 and 11:00 AM, using saplings watered to field capacity. Counting from the top to the bottom, the forth (young) and the seventh (intermediate) leaf were analyzed as well as the second (old) leaf from the base to the apex. The average values for air temperature, relative humidity and CO2 concentration in the chamber were 28,9 ^{0}C, 70% and 369 ppm CO_2, respectively.

The leaves of 'Paluma' used for analysis of antioxidative capacity were those used previously for the gas exchange measurements. For analysis of SOD activity, leaf samples were homogenized in 50 mM phosphate buffer at pH 7.5 (containing 1 mM titriplex III EDTA - Na2, 50 mM NaCl and 1 mM ascorbate) and 2% polyvinylpoly - pyrrolidone (PVPP). After centrifugation, EDTA, phosphate buffer (0.1 M pH 7.0), methionine, nitro - blue tetrazolium (NBT) and riboflavin were added to extract before exposing them to fluorescent light (80 W) for 15 minutes (Osswald *et al.*, 992). Control extracts were maintained in darkness. Absorbance was measured in both illuminated and non - illuminated extracts using a spectrophotometer (= 560 nm) and the differences between both absorbancies were used to determine the SOD activity. The content of total AA was determined for leaf samples homogenized in titriplex III EDTA - Na2 and oxalic acid, followed by centrifugation, addition of 2.6 dichlorphenol - indophenol sodium salt dihydrate, measurement of absorbance at 520 nm, addition of one drop of 1% ascorbate and further measurements of absorbance at 520 nm. The content of AA was estimated based on the differences between the two values of absorbance, according to Keller & Schwager (1977).

The results for foliar injuries, gas exchange and antioxidants obtained for 'Paluma' saplings exposed at both experimental sites (Ibirapuera and the greenhouse) were not directly compared. Thus, the responses of the plants kept in the greenhouse were considered as those expected in 'Paluma' plants maintained in stress - free environment. In contrast, the results for plants from Ibirapuera or the Botanical Institute were assumed to be representative of those induced by the prevailing environmental conditions which, at these sites, include exposure to ozone. All data were tested for normality and variance. Then, differences in analyzed variables different - aged leaves from the same site were tested by analysis of variance (One Way Anova). When Anovas showed differences in the mean, discrimination was confirmed by the Student - Newmann Keuls test. The coefficient correlation were also calculated with the aid of the program SigmaStat3.1 (SYSTAT Software Inc.).

RESULTS AND DISCUSSION

The second exposure period was the most critical one because of the highest maximum hourly concentration of O3 and more frequent exceedances of Brazilian air quality standards. The third exposure was the less critical. For other pollutants (NO2 and MP10) low average hourly concentrations were recorded. In the greenhouse the average concentration of all the monitored pollutants was also negligible. During the exposure period, the mean air temperature varied from 19 ºC to 26 ºC and relative humidity from 79 % to 83 % in Ibirapuera Park and at the Botanical Institute. In the greenhouse, the average temperature was 24.8 ºC and the relative humidity was79%.

Visible foliar symptoms were apparent during all exposure periods, but the elapsed time until their appearance varied from nine days (third exposure - IBt) to 73 days (second exposure-Ibirapuera Park). The highest incidence of foliar symptoms occurred during the first and third exposures; severity was greatest during the third exposure. Plants grown in the greenhouse never showed O_3 - induced foliar injury.

'Paluma' saplings exposed in Ibirapuera Park showed a decreasing mean rate of Asat with increasing leaf age. However, in the greenhouse there were no significant differences of Asat between leaves of different age during the first exposure. In the second and third exposures, the variation in the Asat was similar between saplings exposed to high concentrations of O3 and those which were kept under filtered air; thus, the oldest leaves exhibited significantly smaller photosynthesis rates than young leaves. However, intermediate and old leaves of plants exposed to O3 exhibited greatest reductions in Asat than the corresponding young leaves, with exception of the second exposure. Although old leaves exhibited smaller stomatal conductance during the first exposure, for the other exposures there were no differences between leaves of different age.

Mean AA concentrations were significantly higher in the old leaves of 'Paluma' saplings kept in Ibirupuera Park during the first and the second exposures, but the leaves of plants exposed at Ibirapuera or Botanical Institute during the third exposure did not differ with respect to this variable. 'Paluma' saplings grown in the greenhouse exhibited similar AA concentrations for all leaf age categories and exposures. Comparisons of the concentrations of ascorbic acid from saplings exposed at Ibirapuera revealed that these AA concentrations were significantly higher for all leaf age categories in the second exposure, respectively 14.6, 12.4 and 15.6 mg g DW - 1. The first exposure showed intermediate values (8.3, 9.4 and 14.2 mg gDW - 1), while the third exposure provided the lowest values (3.9, 3.5 and 4.7 mg g DW

- 1), respectively for young, intermediate and old leaves. In general, saplings kept in the greenhouse had lower AA values than those grown in Ibirapuera for the first two exposures. Mean activity of SOD was similar in the 'Paluma' saplings exposed in the filtered air and under ozone polluted areas, despite the high variability between the samples. The values achieved in the first exposure have been exhibited an increase in the activities of SOD enzyme in the intermediate and old leaves of saplings exposed in Ibirapuera Park and in old leaves of saplings kept in greenhouse. On the follow exposures there were no significantly differences in SOD activity in the different leaves analyzed.

4-Discussion

CETESB data indicate that, in the metropolitan region of São Paulo the lowest ozone concentrations occur in winter as a consequence of the lower temperatures and solar intensity. The highest occur in the spring and summer. The concentrations recorded during the study period corroborate this pattern, because the highest value coincided with these seasons, i.e. during the first and second exposures, respectively (CETESB, 2007).

The ozone - induced foliar symptoms in P. guajava 'Paluma' were similar to those previously described for this cultivar (Furlan *et al.*, 007) and also for those reported in previous studies (Sanz *et al.*, 002). The variations in the onset of visible foliar injuries during the three exposures reflect the differing climate and the air quality characteristics during each exposure. The meteorological conditions to which plants are exposed may increase or decrease pollutant uptake and, thus, determine the time required for the O3 uptake dose necessary to induce foliar injury. Thus, the variation in the time required for the manifestation of the visible foliar symptoms is possibly a consequence of the influence of the differing climatic conditions during each exposure.

Exposure to O3, through increases in oxidative stress, reduces carbon assimilation by decreasing the activity and quantity of the enzyme Rubisco (ribulose - 1,5 - biphosphate carboxylase/oxygenase; Pell *et al.*, 994). As a consequence of increasing the degradation of Rubisco and of reducing its synthesis (at a transcriptional and post - transcriptional levels), ozone may be expected to cause more damage in the oldest leaves because they naturally have lower protein synthesis. Such damage may have occurred in the 'Paluma' saplings during the first exposure as the Asat values for old leaves were significantly smaller in the saplings exposed in the Ibirapuera, whereas saplings from the reference greenhouse environment exhibited similar Asat values for all leaf age categories. Nali *et al.*, (2004) identified a positive linear correlation between the reduction in Asat and gs and concluded that the inhibition of carbon fixation could be attributed mainly to the stomatal component. In the present study, the reductions in Asat and gs were also positively correlated in saplings exposed at Ibirapuera during the first and second exposures and in the saplings exposed in the greenhouse during all three exposures periods. Similar results were presented by Oredonvici - Best *et al.*, (2008) for Prunus serotina and Populus maximowizii x trichocarpa. These workers found a strong positive linear correlation between stomatal conductance and carbon assimilation. However, such relation was not verified in the third exposure, which has similar Asat values for young, intermediate and old leaves. This decoupling between Asat and gs could indicate loss of control of the stomatal movements, which disables the plant to avoid the uptake of the pollutant.

Many studies related high concentrations of apoplastic AA to increased O3 resistance. Nevertheless, the correlation between Asat and AA in the present study shows that AA concentrations were greatest in leaves with the lowest Asat values ($r = -0.62$ and $p = 0.001$). Hence, it is supposed that, even though 'Paluma' responded by increasing the AA concentration, the action of this antioxidant might have been insufficient to restrain the oxidative stress. The photosynthetic apparatus may have been damaged before antioxidant concentration had reached a level which has high enough for protective capacity. Moreover, the regeneration of ascorbic acid through the Halliwell - Asada cycle have been low due to the low dynamic efficiency of this enzymatic system. This relation was not observed in the 'Paluma' saplings kept in the greenhouse ($r = 0.26$ and $p = 0.25$).

The SOD enzyme reacts with superoxide radicals to produce hydrogen peroxide and it's activity may be increased in leaves exposed to ozone, contributing to their higher resistance (Nali *et al.*, 004), although some authors report no variation (Moraes *et al.*, 006). In the present study, SOD response was not clear, because it was expected that the increase in enzyme activity in old leaves of saplings exposed to O3 would be more intense than in old leaves of the plants kept in filtered air, instead of reflecting only the aging effect. The hypothesis tested that the old leaves of plants exposed to O3 would have lower Asat values and antioxidants concentrations and therefore exhibit greater visible injury, could not be totally accepted based on the results obtained with the antioxidants, mainly the results of SOD. The results obtained seem to indicate the existence of other antioxidants acting to combat the stress caused by O_3. Anthocyanin is a flavonoid which has amongst other functions, that of neutralizing active species of oxygen and increasing antioxidant activity. Furlan *et al.*, (2008) verified an increase in the quantity of phenolic compounds in the leaves of 'Paluma' after fumigation with O_3. It is suspected that the reddish pigments accumulated in the old leaves of 'Paluma' following exposure to ozone are also anthocyanin. In this manner, its synthesis and accumulation would constitute an additional defense line, or even the major defense, to oxidative stress caused by O3. Therefore, the manifestation of red dots verified in the leaves of 'Paluma' would reflect a form of protection rather than a form of injury.

CONCLUSION

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