

Structural responses of *Ipomoea nil* (L.) Roth 'Scarlet O'Hara' (Convolvulaceae) exposed to ozone

Bárbara Bâesso Moura^{1,2}, Sílvia Ribeiro de Souza¹ and Edenise Segala Alves¹

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RESUMO

(Respostas estruturais em *Ipomoea nil* (L.) Roth 'Scarlet O'Hara' (Convolvulaceae) exposta ao ozônio). Devido à sua alta reatividade e fitotoxidez, o ozônio troposférico (O_3) é um dos mais importantes poluentes fotoquímicos, podendo provocar alterações microscópicas em plantas antes do aparecimento de sintomas visíveis. O presente estudo teve como objetivo aprimorar os conhecimentos sobre os efeitos do O_3 em folhas de *Ipomoea nil*. Plantas foram expostas na cidade de São Paulo em local com altos níveis de O_3 , e também fumigadas com esse gás. Plantas, sem sintomas visíveis, expostas no ambiente, apresentaram alterações microscópicas indicativas do estresse oxidativo como resposta semelhante à de hipersensibilidade (HR-like) e presença de protruções nas paredes celulares; na presença de sintomas visíveis, ruptura da epiderme e colapso do parênquima paliádico e lacunoso. Os experimentos de fumigação confirmaram que tais danos microscópicos foram decorrentes do O_3 . Os resultados mostraram que *Ipomoea nil* responde ao estresse induzido pelo O_3 , apresentando alterações estruturais que antecedem os sintomas visíveis.

Palavras-chave: HR-like, ozônio, protruções de parede celular, danos microscópicos

ABSTRACT

(Structural responses of *Ipomoea nil* (L.) Roth cv. 'Scarlet O'Hara' (Convolvulaceae) exposed to ozone). Troposphere ozone (O_3) is an important photochemical pollutant due to its high reactivity and phytotoxicity. Plants can show microscopic damage caused by this gas before the detection of visible symptoms. The present study aimed to improve our knowledge concerning the effects of O_3 on *Ipomoea nil* L. cv. Scarlet O'Hara leaves. Plants were exposed at a pollutant site in São Paulo city, with high levels of O_3 , and fumigation with O_3 was also carried out. In plants exposed to the environment, without visible symptoms, hypersensitive response-like (HR-like) and protrusions in the cell walls was observed, indicating oxidative processes; in leaves with visible symptoms, there is epidermis breakdown and the palisade and spongy parenchyma collapse. Fumigation confirmed that microscopic damage occurring in the plants exposed to the environment was linked to the symptoms caused by O_3 . These results demonstrate that *Ipomoea nil* responds to stress induced by O_3 with specific structural changes that precede visible symptoms.

Key words: ozone, HR-like, cell wall protrusions, microscopic damage

Introduction

The troposphere ozone (O_3) is a phytotoxic secondary pollutant of easy diffusion, being the most important, damaging, and oxidant among the photochemical pollutants (Klumpp *et al.* 2001; Krupa *et al.* 2001). It enters the leaf through stomata and immediately decays into different reactive oxygen species (ROS), leading to an oxidative burst which might be related to the formation of lesion points as observed in sensitive species (Günthardt-Goerg & Vollenweider 2007). Also, ROS might accumulate in the subcellular compartments of palisade cells, reacting with the lipid membranes towards the initial oxidative

stress, damaging proteins and leading to a physiological deficiency (Schraudner *et al.* 1997). Finally, ROS cause a fast and localized cell death, characterized by cell collapse, an incomplete degradation of the cellular components and a subtle loss of membrane integrity (Günthardt-Goerg *et al.* 1997). According to Günthardt-Goerg & Vollenweider (2007) such effects not only have come out by ozone stress but preferably by pathogen-induced response. So, the induction of plant defense reactions triggered by ozone stress is similar to those induced by pathogens (Rao *et al.* 2000; Sandermann *et al.* 1998). Such reactions include the induction of polyphenols, pathogenesis related proteins

¹ Instituto de Botânica de São Paulo, São Paulo, SP, Brazil

² Author for correspondence: bmourabio@gmail.com

(PR1), cell wall modifications and localized cell collapse and death, which are indicative of programmed cell death (PCD) described as hypersensitive response (HR), which occurs in a discrete groups of cells (Niderman *et al.* 1995). This kind of response can be recognized by structural changes indicated by microscopic analysis in which the stress response can be seen before any visible damage (Günthardt-Goerg & Vollenweider 2007).

Ipomoea nil L. 'Scarlet O'Hara' is well-known as sensitive to O₃ (Nouchi & Aoki 1979; Nouchi & Toyama 1988); it presented visible symptoms when submitted to high levels of O₃ (Domingos *et al.* 1998). Since visible symptoms are preceded by microscopic changes (Vollenweider *et al.* 2003), we hypothesized that *Ipomoea nil* exposed to ozone might manifest structural responses being useful to validate the stress induced by O₃. We also aimed to improve the knowledge about *Ipomoea nil*, describing and comparing the microscopic damages in leaves with and without visible symptoms exposed to controlled O₃ levels and at a polluted site in São Paulo city.

Materials and methods

Plant growth

Seeds of *Ipomoea nil* were acquired from a commercial supplier (CN Seeds LTD, www.cnseeds.co.uk). A mixture based on *Pinus* bark (Plantimax-Eucatex™) and thin vermiculite (3:1) was used as the germination substrate. The seedlings developed in transparent plastic boxes (gerbox) until the emergence of the second cotyledonal leaf, when they were transferred to 2 dm³ plastic pots containing the same substrate.

The plants were irrigated by capillarity according to ISO 3957/6 (VDI 2003). All these plants received 100 ml Hoagland nutrition solution, as described in Epstein (1975), weekly, from the transfer to the end of the experiment.

From germination to *in situ* exposures, the plants were kept in a greenhouse, with filter system for particles and gas pollutants and equipped with air conditioning for temperature control. The greenhouse is located at the Botanical Institute of São Paulo, which is situated in the southeast region of São Paulo city (23°38'08"S and 46°38'00"W, Fernandes *et al.* 2002) and was used throughout the experiment as the reference mark for visible and structural symptoms.

The plants were kept under average relative humidity of 66% during the winter and of 72% in the spring; the average temperature was 22°C in the winter and 28°C in the spring, this way simulating the conditions observed in the outdoor environment.

In situ exposure

Ipomoea nil was exposure when the fifth, sixth, and seventh leaves were well-developed, about one month after

sowing. The plants were exposed in the city of São Paulo-Brazil at Ibirapuera Park (23°34'55"S and 46°39'25"W), a site submitted to high levels of O₃ (Environmental Company of São Paulo State - CETESB 2009). During the experimental period, the plants also had adequate irrigation guaranteed by capillarity through nylon strings and received periodic fertilization with Hoagland nutrition solution (Epstein, 1975).

Two exposures were carried out, one in the winter (25 August to 22 September 2006) and the other in the spring (14 November to 11 December 2006) and, in each experiment, 12 pots stayed at the Ibirapuera Park while other 12 pots were kept in the greenhouse with filters to gases and particles - Purafil™. Samples of the leaves 5, 6, and 7 (from the plant base) were collected weekly, according to the methodology suggested by VDI (2003), from three individuals, guaranteeing that samples were taken throughout the exposure period.

O₃ concentration (ppb) was constantly measured at Ibirapuera Park by CETESB as was some meteorological data (e.g. temperature, relativity humid and global solar radiation).

Fumigation experiment

The experiment were performed in closed chambers with dimensions of 85 x 94 x 85 cm (W x D x H) that were composed of a stainless steel structure connected to an apparatus to humidify and mix the air, covered by a film of Teflon™. Artificial light was supply by 400 W metallic vapor lamps and 30 W fluorescent lamps. O₃ used for fumigation was produced by the dissociation of oxygen under an electrical discharge generator (Ozontechnik) as described by Souza and Pagliuso (2008). Input O₃ levels were continuously measured with an Ecotech™ 9810B O₃ monitor and the chambers received equivalent fluxes of air during the fumigation.

Two sets with six plants each were used in this experiment. Each set was placed inside different chambers simultaneously, with continuous flow (25 dm³ min⁻¹/min) for six hours per day during six days. One set received only filtered air (CF) and the other filtered air added with 40 ppb O₃, in the first exposure and 80 ppb O₃ in the second exposure (CF+O₃). Samples of leaves 5, 6, and 7 from three individuals exposed to the different situations describe above were collected.

Visible symptoms

Leaves' visible symptoms were classified according to Krupa (2001) both on fumigated plants and on field experiment. The presence of the visible symptoms was analyzed during the entire experimental period and photographed for characterization.

Microscopic analysis

Six fragments measuring around 2 cm², randomly selected, were cut off from each leaf (5, 6, and 7) according

to the methodology suggested by Kubínová (1994). The samples were fixed under vacuum in formaldeide, glutalaldeide, acetic acid and alcohol (FGAA) according to Lersten & Curtis (1988) and maintained in the fixative for 48 hours to be later stored in 70% ethylic alcohol.

The material was embedded in glycol methacrylate (GMA) (historesin, Leica) in accordance with the manufacturer's recommendations. The 5 µm sections, obtained from Olympus (Cut 4055) rotary microtome, were stained with 0.5% toluidine blue prepared with acetate buffer 0.1M, pH 4.7 (O'Brien *et al.* 1964).

The observations of the leaf sections were performed on Olympus BX41-BFIII microscopy equipped with a system to capture images and semi-automatic measuring system (Image Pro-Express version 4.0.1 software, Media Cybernetics).

Results

Site study

Winter and spring O₃ patterns are shown in Fig. 1. Levels of O₃ were higher in winter than in spring. Some meteorological data of Ibirapuera Park during the exposure period are shown in Table 1. The plants were kept under ideal moisture conditions in both seasons and the average temperature was around 20°C, an atypical situation concerning the historical average for winter, when temperature is normally lower. The average global radiation was also similar in both seasons. The average concentrations of NO_x, O₃ precursors, were higher in winter compared to spring (data not shown), which probably explains the greater formation of the pollutant in the winter.

Visible and structural variations

Plants kept in greenhouse and under filtrated air did not show any visible symptoms (Fig. 2 A). Visible injuries, characterized by intercostal bleaching, were observed on plants exposed in Ibirapuera Park in both seasons, especially in the winter (Fig. 2 B) and also on those fumigated (Fig. 2 C)

Plants maintained in greenhouse showed no damage to the parenchyma cells (Fig. 3 A and B). On the other hand, samples collected from areas of the leaf with visible symptoms showed epidermal rupture and collapse of the parenchyma cells (Fig. 3 C and D). Such symptoms were observed in groups of cells restricted to the areas with visible symptoms in plants exposed at Ibirapuera Park.

In the mesophyll cells of the plants exposed at Ibirapuera Park, alterations indicative of oxidative stress generated by O₃ were similarly observed in leaves 5, 6, and 7 (data not shown). In plants kept in the greenhouse and exposed to filtered air no structural damages were found (Fig. 4 A and B). In leaves without visible symptoms, either in the plants exposed in the winter or in the spring a collapse was observed in isolated cells of palisade parenchyma,

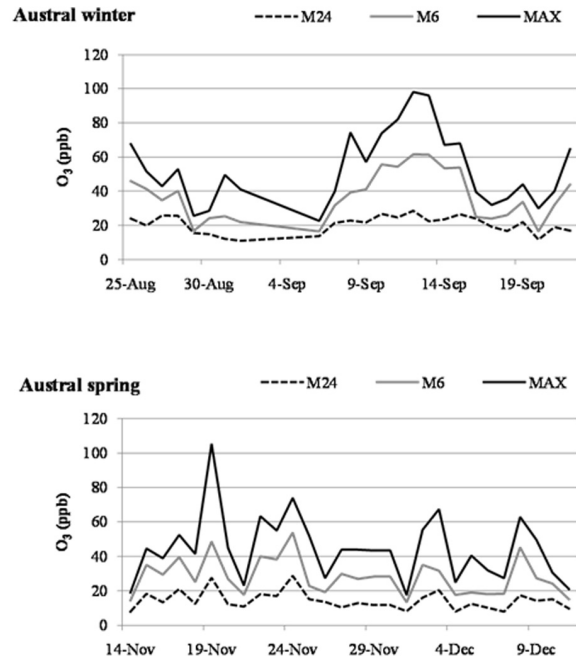


Figure 1. Daily pattern of ground-level O₃ concentration (in ppb) in the two time exposures (austral winter and austral spring) in 2006. M24 = mean of 24h; M6 = mean from 10:00 to 16:00; MAX = maximum daily concentration.

Table 1. Average values of environmental variables at Ibirapuera Park, during both seasons carried out in 2006. Minimum and maximum daily average values are in parentheses. T - temperature. RH - relative humidity. GR - global solar radiation.

	Winter	Spring
T (°C)	19,5 (13,9 - 24,0)	21,6 (19,4 - 25,4)
RH (%)	74 (56 - 90)	82 (64 - 97)
GR (W m ⁻²)	352 (56-488)	181 (77 - 328)

which represents a classic HR-like response, typical of O₃ stress (Fig. 4 C and D). The cell death apparently occurred quickly, since it was possible to detect the presence of chloroplasts even in the necrotic cells. Cell wall protrusions were scattered throughout the photosynthetic tissue mainly in the cells of the spongy parenchyma of plants exposed to field conditions and were not observed in plants from greenhouse (Fig. 5 A and B versus C-F).

Structural variations fumigation

Similarly to the plants exposed in the city of São Paulo, the fumigated plants presented symptoms characteristic of the oxidative stress induced by O₃. Plants fumigated with 80 ppb O₃ showed typical HR-like responses in the leaves, also very similar to those observed in plants exposed in São Paulo, corroborating the symptoms found in the latter (Fig. 4 E). Plants submitted to 40 ppb O₃ did not show visible symptoms, presenting only tortuous cell walls (Fig. 4 F), especially in the palisade parenchyma. In

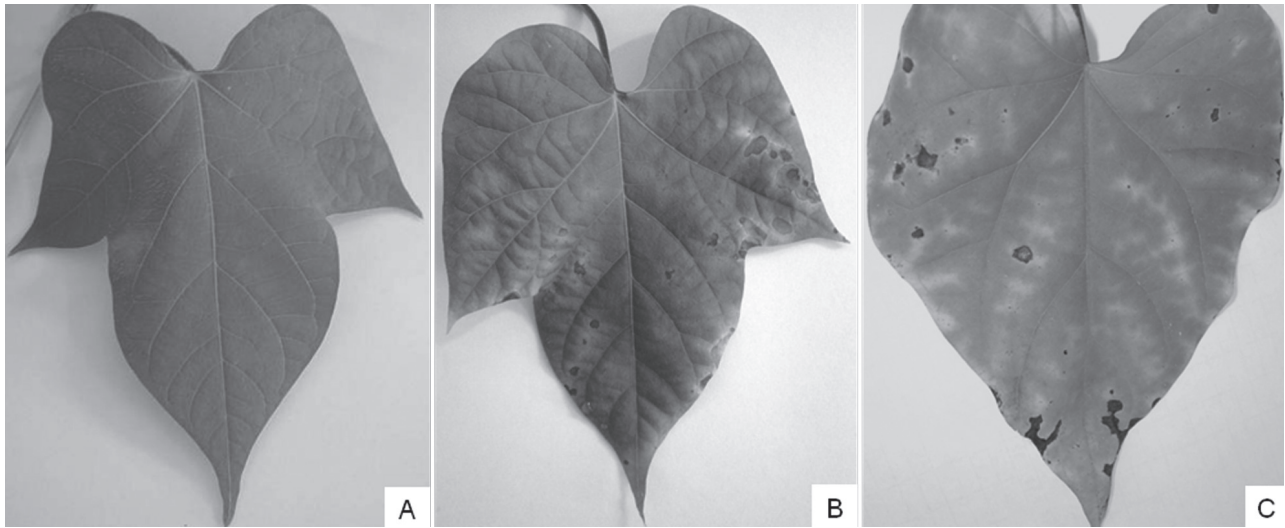


Figure 2. Leaves of *Ipomoea nil*. (A) Reference leaf without visible injury. (B) Visible injury (bleaching) from winter exposure. (C) Visible injury (bleaching) from fumigating experiment (CF + O₃) + 80 ppb.

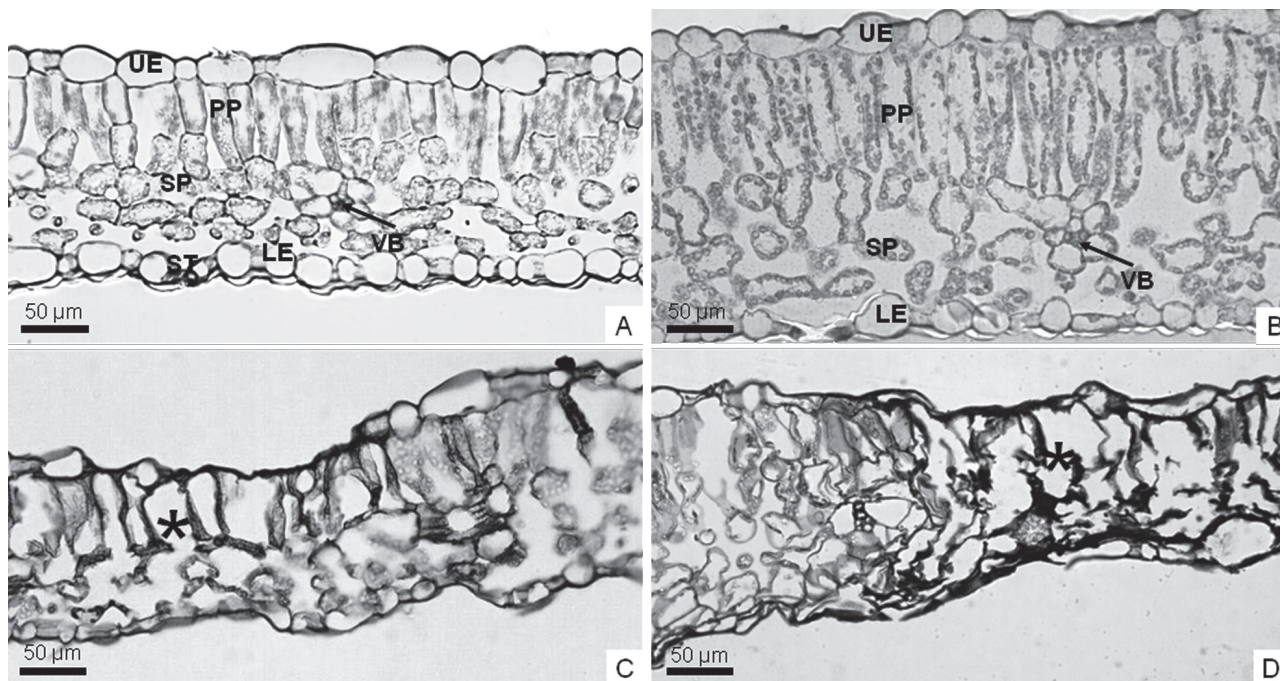


Figure 3. Cross sections of *Ipomoea nil* leaf. (A-B) Plants without symptoms maintained in greenhouse (A Winter, B Spring), (C-D) Plants with necroses (asterisks) maintained in Ibirapuera Park (C Winter, D Spring). UE = upper epidermis, ST = stomata, PP = palisade parenchyma, SP = spongy parenchyma, VB = vascular bundle, LE = lower epidermis.

both experiments it was not possible to find protrusions on the cell wall as those found in leaves exposed to the environment.

Discussion

According to Ferreira *et al.* (2007) *Ipomoea nil* presents an efficient antioxidant defense system which can

protect plants against ozone damage and delay the visible symptoms. However in plants exposed at Ibirapuera Park, independently of the season, and those fumigated with O₃, microscopic damage was observed as discrete groups of cells with remaining organelles and disrupted cell content. This kind of response was described by Günthardt-Goerg & Vollenweider (2007), as a HR-like and it is a type of programmed cell death (PCD) and represents a plant

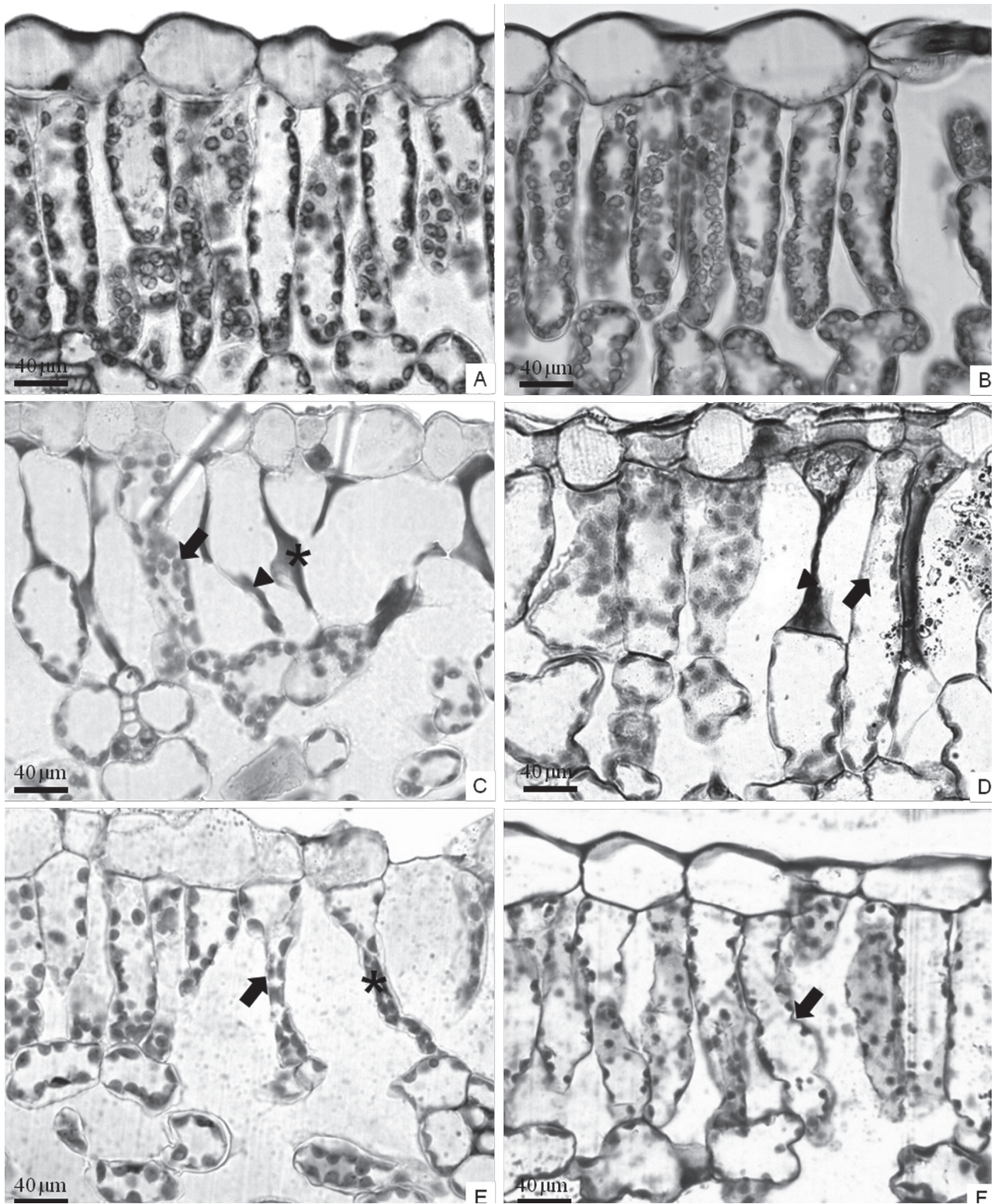


Figure 4. Cross sections of *Ipomoea nil* leaves. (A) Plant maintained in greenhouse without symptoms. (B) Plant maintained in chamber with filtrated air. (C and E) Plants maintained in Ibirapuera Park with a HR-like (asterisks) with remind chloroplasts (arrow) and cell-wall collapse (arrowheads) (C Winter, D Spring). (E) Plant maintained in chamber (+ 80 ppb) with HR-like (asterisks). (F) Plant maintained in chamber (+ 40 ppb) with marked tortuous cell walls.

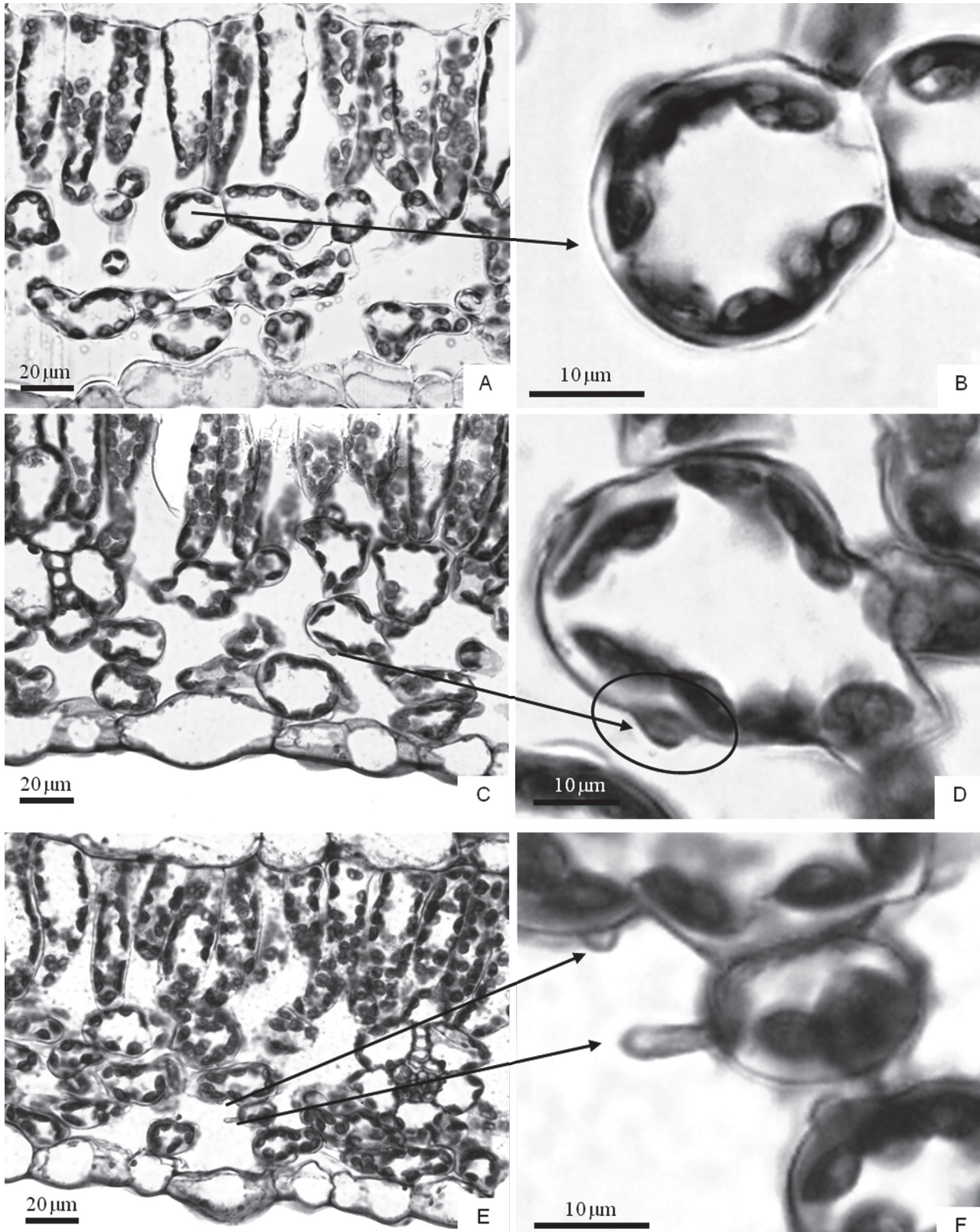


Figure 5. Cross sections of *Ipomoea nil* leaves. (A and B) Plants maintained in greenhouse with normal aspect of cell wall in the spongy parenchyma. (B) Detail. (C - F) Plants maintained in Ibirapuera Park with cell wall protrusions. (D and F) Detail.

defense mechanism against biotic stress, which can also be induced by a few abiotic stress factors. The criteria to identify the HR-like response according to the same authors are the absence of pathogens, restriction of the PCD to a small group of cells, rupture of the cell contents, collapse of the cell walls, and incomplete degradation of the cell as a consequence of fast cell death. Such symptoms were observed in *Ipomoea nil* and turned up being very similar to the results reported by Vollenweider *et al.* (2003) in *Robinia pseudoacacia*.

According to Gravano *et al.* (2003), the HR-like response is normally observed in localized regions, especially in the palisade parenchyma, where it occurs in isolated groups of cells and has been used to corroborate the microscopic damage caused by O₃.

In the *Ipomoea nil* leaves, it was also possible to observe the presence of the protrusions on the cell walls. Such protrusions occurred preferably in the spongy parenchyma cells and were evidenced only in the plants that were kept at Ibirapuera Park.

Several authors mention the presence of protrusions in plants submitted to O₃. Günthardt-Goerg *et al.* (2000) reported the appearance of pectic protrusions and phenolic incrustations in *Prunus serotina* submitted to critical levels of O₃. They also described the presence of cell wall invaginations in the chlorophyll parenchyma, the presence of a condensed cellular content, the cellular collapse even before the reallocation of the cellular contents and the cell death. In *Pistacia lentiscus* collected in an environment submitted to high O₃ concentrations, Reig-Armiñana *et al.* (2004) described the appearance of protrusions on the cell walls of the spongy parenchyma cells and, furthermore, the degeneration of the tonoplast and consequent cellular disorganization. Günthardt-Goerg & Vollenweider (2007) documented the appearance of HR-like responses and protrusions on the spongy parenchyma cell wall in *Sambucus racemosa* as a result of the oxidative stress caused by O₃. The authors affirm that the microscopic alterations can be simultaneous or previous to the first visible symptoms, which is of interest, since it allows forecasting beforehand plant sensitivity to O₃.

Also, no alterations were observed in the conduction tissue of *Ipomoea nil*, neither in the plants exposed São Paulo, nor in the fumigated plants, indicating that the symptoms induced by O₃ are occurring between the vascular bundles and not over them (Novak *et al.* 2003) as a characteristic of the O₃ stress.

Studying the leaf samples with visible symptoms of *Ipomoea nil* exposed at Ibirapuera Park, a necrosis through a part or all through the leaf blade was found, both in the palisade and spongy parenchyma as well as in the epidermis cells perceiving in an isolated region of the leaf. With the progressive increase of visible damage, the cells appeared empty with invaginated cell walls and condensed cell content.

In general, the symptoms observed in *Ipomoea nil* exposed in the city of São Paulo were the same described by the different authors herein discussed. Furthermore, the symptoms observed in the plants fumigated with 80 ppb O₃ were similar to the symptoms present in the *in situ* exposed plant, which allows its validation. Vollenweider & Günthardt-Goerg (2006) affirm that, in the validation of the damage caused by abiotic agents, a group of markers should be sought. Much attention should be paid to the localization of the symptoms in the affected cell tissues and microscopic and physiological responses as well as visible symptoms should be combined in order to achieve a better diagnosis of the stress induction agent.

In conclusion it was possible to observe in *Ipomoea nil* microscopic damages caused by O₃ as HR-like, indicating that ozone-induced response might be detected by specific structural changes that precede the visible symptoms.

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