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Synergism between ozone and light stress: structural responses of polyphenols in a woody Brazilian species

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HIGHLIGHTS

- The ozone-light stress affects the distribution of polyphenols on leaf tissues.
- Aspects of stress-induced polyphenols were compared to those from secretory glands.
- Dense aspect of polyphenols precedes PCD in constitutive and stress-induced defenses.
- There is homology of process between polyphenols from chlorenchyma and glands.

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ABSTRACT

Microscopic studies on isolated ozone (O_3) effects or on those in synergy with light stress commonly report the induction of polyphenols that exhibit different aspects within the vacuole of photosynthesizing cells. It has been assumed that these different aspects are randomly spread in the symptomatic (injured) regions of the leaf blade. Interestingly, secretory ducts that constitutively produce polyphenols also exhibit these same variations in their vacuolar aspect, in a spatial sequence related to the destiny of these cells (e.g., programmed cell death (PCD) in lytic secretion processes). Here, we demonstrate that the deposition pattern of polyphenols prior to the establishment of the hypersensitive-like response, a type of PCD caused by O_3, follows the same one observed in the epithelial cells of the constitutive lysigenous secretory ducts. *Astronium graveolens*, an early secondary Brazilian woody species, was selected based on its susceptibility to high light and presence of secretory ducts. The synergism effects were assessed by exposing plants to the high O_3 concentrations at an urban site in São Paulo City. Confocal, widefield and light microscopies were used to examine polyphenols' occurrence and aspects. The spatial pattern of polyphenols distribution along the leaflets of plants submitted to the synergism condition, in which a dense vacuolar aspect is the target of a cell destined to death, was also observed in the constitutive secretory cells prior to lysis. This similar structural pattern may be a case of homology of process involving both the constitutive (secretory ducts) and the induced (photosynthesizing cells) defenses.

1. Introduction

Excessive solar radiation (harmful UV and excessively absorbed visible quanta) strikes the plant photosynthetic apparatus and exposes the chloroplasts to an energy excitation surplus that causes intense formation of reactive oxygen species (ROS). ROS activate enzymatic and non-enzymatic antioxidants, such as polyphenols, to combat photooxidative damages (Agati et al., 2012, 2013; Fini et al., 2012), especially in species with greater light sensitivity. *Astronium graveolens*, an early secondary Brazilian species, exhibit larger reductions in daily-integrated CO_2 assimilation and severe damages when transferred from medium light (34% of full sunlight, optimum for this species) to high irradiance (Ribeiro et al., 2005; Assis, 2014).

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Oxidative damages in the photosynthesizing tissues may be intensified by the synergism between intense light conditions and ozone (O_3) exposure (Foyer et al., 1994; Vollenweider et al., 2003; Bussotti, 2008). This synergism leads to responses such as polyphenols oxidation, accelerated cell senescence and hypersensitive responses similar to those caused by some microorganisms but triggered by abiotic stress, named HR-like (Vollenweider et al., 2003; Paoletti et al., 2009; Moura, 2013). HR-like is a particular form of programmed cell death (PCD) restricted to discrete groups of photosynthesizing cells, which exhibit fast alterations in polyphenols composition and oxidation, incomplete organelle degradation, rupture of cell contents, collapse of cell walls, and condensation of cell remnants to apoptotic like bodies (Vollenweider et al., 2003; Günthardt-Goerg and Vollenweider, 2007; Faoro and Iriti, 2009).

During light stress, the excess of excitation energy generates ROS and decreases the ascorbate peroxidase activity inside the chloroplasts, thus causing the diffusion of ROS to the outside of these organelles (Agati et al., 2012). Some flavonoids such as quercetin, a compound found in *A. graveolens* leaflets (Silva et al., 2011; summarized in Table 1), may limit this diffusion by delaying the signals that trigger PCD (Agati et al., 2012). Glycosylated flavonols derivatives (less toxic to the plant) are found in the apoplast, cell wall, chloroplast membrane and inside the vacuole of photosynthesizing cells of healthy plants (Endo et al., 2012; Petrussa et al., 2013). These compounds are found in low amounts (constitutive defenses), often not detectable by routine analytical techniques (Agati et al., 2013). The induction of their synthesis, however, is stimulated by visible light, thereby facilitating the fluorescence analysis (Agati et al., 2012, 2013).

Tropospheric O_3, an atmospheric pollutant, may also trigger the induction of polyphenols (Furlan et al., 1999, 2010; Bussotti et al., 2005; Bartoli et al., 2013). O_3 exhibits stronger oxidizing power than other gaseous pollutants (Krupa et al., 2001), positively correlating with polyphenols metabolism changes (Biolley et al., 2002; Sandre et al., 2014). The visible injuries (such as stippling), which are a specific feature of the oxidative stress caused by O_3 in the leaf blades of many species, are caused by cell death (necrosis) and may also be intensified by polyphenols oxidation (Krupa et al., 2001; Rezende and Furlan, 2009; Booker et al., 2012). Flavonols, anthocyanins and condensed tannins stand out as polyphenols usually involved in O_3 detoxification (Vollenweider et al., 2003; Bussotti et al., 2005; Furlan et al., 2007; Tresmondi and Alves, 2011; Guerrero et al., 2013; Moura, 2013; Moura et al., 2014). To our knowledge, alterations concerning hydrolysable tannins (the ones found in *A. graveolens* leaflets) in response to O_3 have not yet been reported.

Constitutively, tannins are confined within the vacuole of idioblasts or lumen of secretory ducts or cavities due to their high cell toxicity (Castro and Demarco, 2008). The secretory ducts of *Astronium graveolens* produce an oil-resin exudate composed of polyphenols (Table 1) and terpenoids (Hernández et al., 2013). These compounds are synthesized by epithelial cells that constitute the secretory ducts and are released into the lumen by the lysis of these same cells (holocrine secretion – Chen and Wiemer, 1984; Carmello-Guerreiro and Paoli, 2000; Silva et al., 2011). Regarding the phenolic fraction of this secretion, Joel and Fahn (1980a,b) described distinct aspects of the vacuolar content of epithelial cells of *Mangifera indica* (Anacardiaceae) along their ontogenesis, thus culminating in a dense aspect filling the vacuole at the last stage (prior to lysis).

Table 1
Major polyphenols identified by Silva et al. (2011) from *Astronium graveolens* leaflets extract. Note the absence of condensed tannins (proanthocyanidins).

Flavonoids	Hydrolysable tannins and their precursors
Quercetin	Gallic acid
Quercetin-*O*-pentoside	Ellagic acid
Quercetin-*O*-deoxy-hexoside	Ellagitannins
Miricetin-*O*-deoxy-hexoside	
Quercetin-galoyl-pentoside	
Quercetin-galoyl-*O*-deoxy-hexoside	
Quercetin-galoyl-hexoside	

Tannin synthesis can be also induced by abiotic stresses (Ramakrishna and Ravishankar, 2011). In oxidative stress situations caused by excessive light and/or O_3, condensed tannins exhibiting different aspects are visualized within the vacuole of distinct photosynthesizing cells (Bussotti et al., 1998; Reig-Arminñana et al., 2004; Kivimäenpää et al., 2014). These variable aspects may be related to the occurrence of different classes of polyphenols inside the vacuole of distinct photosynthesizing cells when the same plane of the leaf section is observed (Franceschi et al., 1998). These aspects may also be related to different polymerization degrees of polyphenols inside the vacuole of a single photosynthesizing cell, as a response to the synergism between O_3 and light stresses (Vollenweider et al., 2003). However, the existence of some spatial pattern (i.e., three-dimensional alterations concerning symptoms occurring along the leaf blade) that explains the establishment and aspect of polyphenols inside photosynthesizing cells has not yet been assessed.

During our analyses concerning morphological symptoms of O_3 damages from *A. graveolens* plants exposed to field conditions, microscopic alterations identified within stippling areas contrasted with previous results from both chronic and acute fumigations of this species (Moura, 2013; Moura et al., 2014). Such contrast may be a result of the exposure of plants to the synergism between O_3 and light stress. Here we present a detailed structural study concerning the constitutive polyphenols-producing glands (lysigenous secretory ducts) and the induced-polyphenols occurrence inside photosynthesizing cells in response to both O_3 and light stress. We hypothesized the existence of homologous structural processes between constitutive and induced defenses. We compare the obtained data to the structural alterations observed in both acute and chronic fumigations (Moura, 2013; Moura et al., 2014), highlighting the alterations caused in response to synergism.

2. Material and methods

2.1. Plant material and treatments

Seedlings (approx. 20 cm high) were purchased from BIOflora Company (São Paulo, Brazil) and transplanted into 20 L pots filled with 3:1 peat and sand mixture. They were watered by capillarity, and fertilized with Peters solution (10:10:10) each 15 days. Plants were kept inside a greenhouse (with filtered air) at Instituto de Botânica (São Paulo, Brazil) during three weeks for acclimation. The aluminum support where the plants were kept on, either inside the greenhouse or in the field was covered with a shading screen that allowed 65% of sunlight passage to avoid excessive light stress. PAR inside the greenhouse was estimated by measuring the solar radiation inside and outside it every two hours (Assis, 2014). The incidence attenuation estimated was about 30%, i.e. PAR inside the greenhouse corresponds to 70% of full sun radiation measured. This data was obtained during the same period when our exposures were carried out. Samples in the field received 65% of sunlight due to the use of the shading screen, while those inside the greenhouse received 35%. A weather station (WatchDog, Spectrum, IL, USA) was installed inside the greenhouse to register humidity and temperature data.