BÁRBARA BAÊSSO MOURA

Análises estruturais e ultraestruturais em folhas de espécies nativas sob influência de poluentes aéreos

Tese apresentada ao Instituto de Botânica da Secretaria do Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

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ORIENTADORA: DRA. EDENISE SEGALA ALVES

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“Imagination is more important than knowledge”

Albert Einstein

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Resumo

Os efeitos de concentrações tóxicas de ozônio troposférico (O_3) sobre a vegetação natural têm sido relatados, principalmente para os ecossistemas do hemisfério norte com clima mediterrânico temperado ou boreal. No hemisfério sul, em especial nos ecossistemas tropicais, os efeitos do O_3 continuam pouco conhecidos, enquanto os níveis de poluição do ar estão aumentando em consequência do crescimento das economias emergentes. A Região Metropolitana de Campinas (RMC), em São Paulo, Brasil, experimenta altos níveis de O_3 troposférico que podem ser prejudiciais para a vegetação local. O objetivo deste estudo foi investigar os efeitos das concentrações de O_3 em três espécies de árvores nativas: *Astronium graveolens*, Anacardiaceae, *Piptadenia gonoacantha*, Fabaceae e *Croton floribundus*, Euphorbiaceae ocorrentes em fragmentos de floresta semidecidual, localizados na RMC. Experimentos controlados para induzir a formação de sintomas visuais específicos foram realizados, nos quais mudas foram expostas ao ar enriquecido com O_3, em câmaras fechadas. *A. graveolens* e *P. gonoachanta* desenvolveram "stippling" e pontuações, respectivamente, como reações específicas quando expostas ao O_3, enquanto que *C. floribundus* não apresentou nenhuma reação específica. Marcadores microscópicos validaram os sintomas encontrados em amostras coletadas nos fragmentos florestais da RMC. Assim como no hemisfério norte, a vegetação de ambientes tropicais do sudeste do Brasil também esta sendo afetada por concentrações tóxicas de O_3, que contribuem para a degradação destes poucos fragmentos florestais que possuem alta biodiversidade.

Abstract

Toxic effects of current tropospheric ozone (O_3) concentrations on the natural vegetation have been reported primarily for ecosystems in the northern hemisphere with mediterranean, temperate or boreal climate. In the southern hemisphere and tropical ecosystems, the effects of O_3 still remain little known whilst the air pollution is increasing as a consequence of the sustained growth within emerging economies. The Metropolitan Region of Campinas (MRC) in São Paulo, Brazil, presents high levels of tropospheric O_3 which can be harmful to the local vegetation. Aims in this study were to investigate the effects of O_3 concentrations in three native tree species: *Astronium graveolens*, Anacardiaceae; *Piptadenia gonoacantha*, Fabaceae and *Croton floribundus*, Euphorbiaceae from semidecidual forest fragments located in the MRC. Controlled experiments were performed where seedlings were exposed to O_3-enriched air using indoor chambers to induce the formation of specific visual symptoms. *A. graveolens* and *P. gonoachanta* developed stippling and mottles respectively as specific reactions to O_3 exposure and *C. floribundus* did not present a specific O_3 reaction. Microscopical marks validated the symptoms found in samples collected in forest fragments in the MRC. As in north hemisphere, tropical environments in southern Brazil are also being affected by ambient O_3 toxic concentrations which contribute to the degradation of these forest fragments that have high biodiversity.

Índice

Apresentação

Uma introdução geral inicia a tese, na qual são abordados os aspectos gerais sobre a importância dos estudos sobre o efeito do ozônio (O_3) em plantas. Também são apresentados aspectos relevantes para validação dos sintomas visíveis causados pela exposição ao O_3 e são discutidos os mecanismos de sensibilidade de espécies vegetais aos efeitos oxidativos desse gás. A hipótese e os objetivos gerais são destacados ao término da apresentação.

Após a introdução geral, a tese esta dividida em três capítulos, sendo que dois deles foram redigidos em inglês, não apenas para acelerar a publicação dos artigos como, também, para possibilitar a comunição com os colaboradores estrangeiros que contribuíram para a realização deste estudo. A formatação dos capítulos seguiu o modelo das respectivas revistas às quais serão submetidos, contudo, as presentes versões serão revisadas pelos demais colaboradores e por especialista na língua inglesa, antes dos artigos serem submetidos à publicação.

No primeiro capítulo a região de estudo foi caracterizada com relação aos fatores climáticos e concentrações de O_3 as quais a vegetação está submetida. As informações reunidas no Capítulo 1 foram importantes para entendermos as respostas das espécies estudadas, apresentadas nos Capítulos 2 e 3. O artigo resultante do Capítulo 1 será submetido ao periódico Atmospheric Environment.

O segundo capítulo trata da validação dos sintomas visíveis provocados pela exposição ao O_3, com base em marcadores estruturais e ultraestruturais, nas três espécies investigadas. O artigo resultante do Capítulo 2 será submetido ao periódico Environmental Pollution.

Para compreender melhor a sensibilidade das espécies estudadas, o terceiro capítulo aborda os aspectos histoquímicos e ultraestruturais responsáveis pela indução dos sintomas visíveis. O artigo resultante do Capítulo 3 também será submetido ao periódico Environmental Pollution.

Esclarecemos que os artigos a serem submetidos à publicação possuem coautores que colaboraram com o estudo, contudo, como os mesmos ainda não tiveram acesso às versões aqui apresentadas, optamos por não citá-los na tese. A tese é concluída com as considerações finais sobre os resultados encontrados e a possibilidade de estudos futuros.

Introdução geral

O O_3 e seus efeitos sobre a vegetação

O ozônio (O_3) é a forma triatômica do oxigênio e sua existência na troposfera é conhecida desde 1840 (Percy et al., 2003). Embora na estratosfera seja um importante componente na proteção contra os raios ultravioletas, na troposfera é um dos mais notórios poluentes aéreos (Jasper et al., 2005).

O mais importante mecanismo de formação do O_3 na superfície terrestre é decorrente do ciclo fotoquímico. As suas substâncias precursoras, hidrocarbonetos voláteis (HCs) e óxidos nítricos (NO_X), provêm de processos naturais e biológicos, mas, principalmente, da queima de combustíveis fósseis (Sawyer et al., 2000).

A concentração troposférica de O_3 vem aumentando principalmente em regiões metropolitanas industrializadas (Percy et al., 2003). Há um século, os níveis basais de O_3 não ultrapassavam 15 ppb, sendo que hoje estes níveis atingem 40 ppb (Finlayson-Pitts e Pitts, 2000).

Uma vez que existe um grande número de espécies vegetais sensíveis aos efeitos do O_3 (Percy et al., 2003), calcula-se que 50% das áreas florestais do mundo podem estar em risco devido aos danos oxidativos provocados pela exposição ao poluente (Shriner e Karnosky, 2003). Modelos globais de previsões da exposição de florestas ao O_3 (Fowler et al., 1999;. Derwent et al., 2002) indicam que a vegetação vem sofrendo com os efeitos fisiológicos, que causam a diminuição do crescimento de espécies nativas ou de culturas agrícolas.

Estima-se que 90% da perda em culturas produtivas dos Estados Unidos da América (EUA) se deve à exposição das plantas ao O_3, isoladamente ou em combinação com outros poluentes aéreos, e acredita-se que os mesmos valores de perda de produtividade sejam válidos para as florestas dos EUA (Karnosky et al., 2003).

Embora grande ênfase venha sendo dada aos estudos sobre os efeitos do O_3 sobre a vegetação de clima temperado do hemisfério norte, no hemisfério sul, as respostas de diferentes espécies tropicais aos efeitos do O_3 é incerta (Sitch et al., 2007). Portanto, a hipótese de que as florestas tropicais também estão sendo afetadas pelos efeitos danosos do O_3 nos motivou a conduzir o presente estudo.

Validação de sintomas foliares visíveis e sensibilidade das plantas aos efeitos do O_3

Os sintomas foliares visíveis e específicos decorrentes da ação oxidativa do O_3, podem ser validados com sucesso por meio de marcadores estruturais (Günthardt-Goerg e Vollenweider, 2007). Desta maneira, com a combinação de marcadores estruturais é possível diferenciar o sintoma provocado pelo O_3 daqueles decorrentes de outros agentes estressores.

A principal entrada do O_3 nas folhas se dá pelos estômatos. Uma vez dentro da folha o O_3 é dissolvido nos fluidos da cavidade subestomática, portanto a concentração de O_3 no interior das folhas é próxima a zero (Noormets et al., 2000), o que significa que o poluente é rapidamente degradado, sendo produzidas espécies reativas de oxigênio - ERO (Mittler et al., 2004). O apoplasto é o primeiro local de ação das ERO, que são consideradas como indutoras das respostas celulares, além de serem capazes de oxidar proteínas, membranas e outros componentes, dentro e fora das células vegetais (Overmyer et al., 2009).

O H_2O_2 é uma importante ERO capaz de se difundir através da membrana plasmática via certos tipos de aquaporinas (Bienert et al., 2007), podendo espalhar o efeito oxidante do O_3 para as demais células (Overmyer et al., 2009).

Os eventos que ocorrem no interior das folhas seguem, segundo Heath (1999), a seguinte sequencia: (1) a rápida entrada do O_3 ativa o sistema de resposta antioxidante das plantas; (2) a permeabilidade, o transporte e os mecanismos desencadeadores de processos metabólicos da membrana são alterados. A taxa de movimento de íons e a sensibilidade às

moléculas sinalizadoras se torna muito lenta ou muito rápida para que a homeostase seja mantida; (3) esta mudança no estado redox ativa sinais de transdução que resultam em um processo similar ao da resposta de hipersensibilidade - HR-like (Jasper et al., 2005); (4) o estresse oxidativo induz a formação de sintomas visíveis específicos; (5) a fixação de carbono e, consequentemente, a produtividade são afetadas.

Com a formação das ERO inicia-se uma cascata de respostas (Jasper et al., 2005) que ativa o sistema antioxidante das plantas, e funciona com um primeiro mecanismo para aliviar o estresse oxidativo, sendo que existe uma correlação positiva entre a tolerância das espécies ao O_3 e sua capacidade antioxidante (Conklin e Last, 1995).

Hipótese e objetivos gerais

Considerando a hipótese de que os altos níveis de O_3 registrados na Região Metropolitana de Campinas-SP/Brasil podem ser tóxicos para a vegetação nativa local, o principal objetivo deste estudo foi buscar marcadores estruturais que possibilitem a validação de sintomas visíveis específicos decorrentes da exposição ao O_3, a fim de inferir sobre a sensibilidade das espécies tropicais aos efeitos oxidativos do O_3.

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Capítulo 1

Ozone distribution and its potential toxicity to seasonal semi-deciduous forest fragments in Southern Brazil

Ozone distribution and its potential toxicity to seasonal semi-deciduous forest fragments in Southern Brazil

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Abstract

The Metropolitan Region of Campinas (MRC), located in state of São Paulo-Brazil, presents a complex discharge of primary air pollutants to the atmosphere that contributes to the formation and accumulation of ozone (O_3) in the area. The O_3 is a toxic pollutant that has been responsible to the decline of forest in tempered climate, but little is known about its effect on tropical environment. The aim of this study was to infer about the impact of O_3 on tropical vegetation and to suggest O_3 indices appropriated to this specific environment. Data from The Environmental Company of Sao Paulo State (CETESB) were used to calculate climate parameters and O_3 indices as SUM00, SUM60 and AOT40 in the MRC. The O_3 in the RMC is formed throughout the year and daily distribution is typical of urban areas. The O_3 levels in the MRC are high enough to cause oxidative stress on the local vegetation which may be contributing to the decline of this important environment and since the local

vegetation is mostly active along the entire year, SUM00 and SUM60 calculated for the whole year round seems to be the best indices to be used in tropical regions.

Introduction

Ozone (O_3) is a toxic secondary pollutant produced photochemically by reactions between primary pollutants, such as nitrogen oxides (NOx = NO + NO_2) and volatile organic compounds (VOC). The main sources of O_3 precursors, NOx and VOCs, are related to anthropogenic activity, especially to fossil fuel combustion. With the industrialization and motorization, evidences suggest that O_3 background levels doubled over the past century in both, north and south hemispheres (Ueda et al., 1988; Sandroni et al., 1992).

The adverse effects of O_3 on plants were first identified in the 1950's and now, this air pollutant is recognized as the most phototoxic one, affecting vegetation, materials and the human health (Ashmore, 2005). An increasing number of reports appeared during the past 25 years regarding O_3-induced foliar injury on sensitive plants in many North Hemisphere countries in European, as in Switzerlad (Novak et al 2003) and Italy (Bussotti and Ferretti 2009) and also in the USA (Vollenweider et al 2013). Nevertheless, there are some studies about the O_3 effects on South Hemisphere environments, most of them based on active monitoring networks, using bioindicator species (Alves et al., 2011; Sant'Anna et al., 2008), but only few considering natural vegetation (Klumpp et al., 2000; Maioli et al., 2008).

Metropolitan Region of Campinas (MRC) is composed of 19 cities, with a population of around 3 million inhabitants representing 15% of the São Paulo state population and the region has 8% of the fleet vehicle of the state. RMC is the region with the greatest industrial and economic expression in the countryside of the state with industrial park major composed of petrochemicals, textile, foodstuff, automobile, metallurgy and pharmaceutical industries.

Besides the emission of pollutants by the industry, the region is also registers an expressive vehicle pollution emission (Ueda and Tomaz, 2011). The air pollution dynamic is complex in the MRC. According to Ueda and Tomaz, (2011), vehicles are responsible, for 99.0, 82.5 and 81.2% of the emissions of CO, hydrocarbons and NOx, respectively, whereas particulate matter emissions are predominantly from industrial sources (fig 1). Moreover the local emission, the transport of pollutants from Metropolitan Region of São Paulo (MRSP) to MRC is favored by the wind south and southwest direction (Boian et al., 2012).

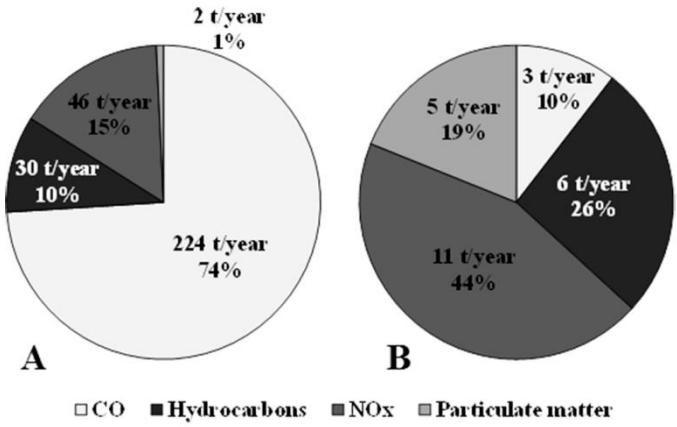

Figure 1. Comparison between vehicular (A) and industrial (B) emissions of RMC. Source; Ueda e Tomaz, (2011).

The vegetation of MRC is classified as sazonal semideciduous forest with more than 450 arborous species (Santin, 1999) that can be divided on three groups: evergreen species (55%) - with leaf drop not concentrated in one period of the year and continuous, intermittent leaf flushing; semi-deciduous species (16%) - with more intense shedding during the dry season, reaching the pick of shedding in July, although never staying with no leaves, with flux of new leaves usually occurring between August and October, during the transition from dry to wet season and deciduous species (41%) - with leaf drop and leaf flushing concentrated during the wet season, remaining without leaves during the dry period (Morellato, 1991). However, the forest current aspect of the vegetation is green and the dynamics of forest foliage renewal are species-specific.

Nowadays, this vegetation is composed of only remain forest fragments very isolated and in an extreme fragmentation processes (Nascimento et al., 1999; Filho and Santin, 2002). A large number of anthropogenic disturbance factors as fires, selective extraction, disposal of waste and rubble (Filho and Santin, 2002) causes a strong impact on MRC vegetation, however, little is known about the toxic effects of O_3, and its potential to cause oxidative stress on this specific vegetation.

Given the great damage that O_3 can cause on the natural vegetation and crops, some indices were adopted to estimate the ozone risk to the vegetation. including the SUM00 (sum of all hourly concentrations in a year without a threshold) and the SUM60 (sum of all hourly concentrations in a year threshold above a threshold of 60 ppb) in the United States of America and the AOT40 (concentration accumulated over a threshold O_3 concentration of 40 ppb) for the United Nations Economic Commission of Europe (UN/ECE, 2004). Those indices are used to map geographic areas where O_3 exceeds critical levels and to established its potential toxicity to the vegetation (Paoletti et al., 2007).

The aim of this study was to characterize the interannual, seasonal and daily pattern of O_3 distribution on tropical environmental, specifically in the MRC, to suggest O_3 indices appropriated to characterize impact probability of this pollutant on tropical vegetation, taking in account the O_3 yearly dynamic, vegetation phenology and climate parameters.

Material and methods

Study site climate and pollution

The climate in the RMC is predominantly subtropical humid classified as Cfa type, according to the Köppen and as B1rB´4a according to Thornthwaite (Rolim et al., 2007). The wet season occurs from October to March (Franchito et al., 2008) with monthly rainfall

higher than 200 mm and average temperature of 24°C. The dry season is between April to September (Franchito et al., 2008) with monthly rainfall around 30 mm and average temperature of 20°C (fig. 2).

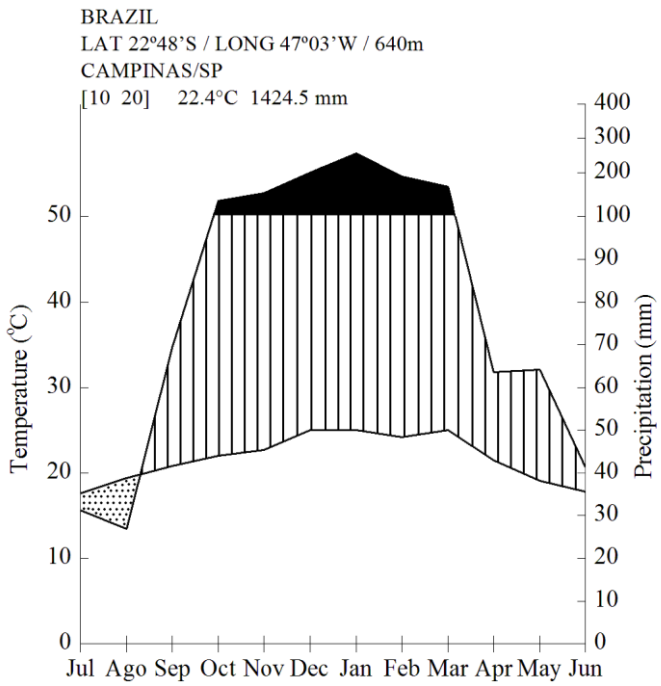

Figure 2. A. Climate diagram summarizing the climatic condition at MRC during the 1988 - 2008 reference periods. Diagram is plotted according to Water and Lieth (1967). Between 0 and 100 mm precipitation monthly, 20 mm on the right ordinate is equal to 10ºC of average temperature on the left ordinate. Above 100 mm, precipitation is plotted using a scale 5 times larger. The wet season are outlined as *solid area* (from October to March) and *dashed* and *dotted* areas out of this period (from April to August) correspond to the dry season. Source: Cepagri (Centro de Pesquisas Meteorológicas e Climáticas Aplicadas à Agricultura) website (http://www.cpa.unicamp.br/outras-informacoes/clima-de-campinas.html).

The Environmental Company of the State of São Paulo (CETESB) maintains an air quality monitoring network with the main objective to target human healthy that allows the assessment of the major pollutants concentrations in the air in different cities around the state of São Paulo. Climate parameters as temperature, humidity, wind direction and solar radiation are monitored such as the pollutants O_3, nitric oxides (NO and NO_2), SO_2 and particulate matter. The three last pollutants had not exceeded standard limits established by CETESB for

São Paulo state since 2009, unlike the O_3 which levels registered in the region surpassed exceeded the air quality standard several times during the past years (CETESB, 2012).

To demonstrate O_3 annual and daily distributions Paulínia's monitoring station (fig 3) date were considered, once this monitoring station has more completed data since 2001. Ozone annual distribution was described considering hour concentration from 8:00 to 20:00. Solar radiation and vapor deficit pressure (VDP) distribution were also considered to show the dynamic of those two parameters along the year and the day, once they are closed related to the O_3 formation and vegetation physiological status, respectively. VPD was calculated by using the automatic calculator by Autogrow System Ltd. (http://www.autogrow.com/downloads/download-software-and-drivers). Daily distribution of NO and NO_2 were also used to understand the O_3 dynamic along the day.

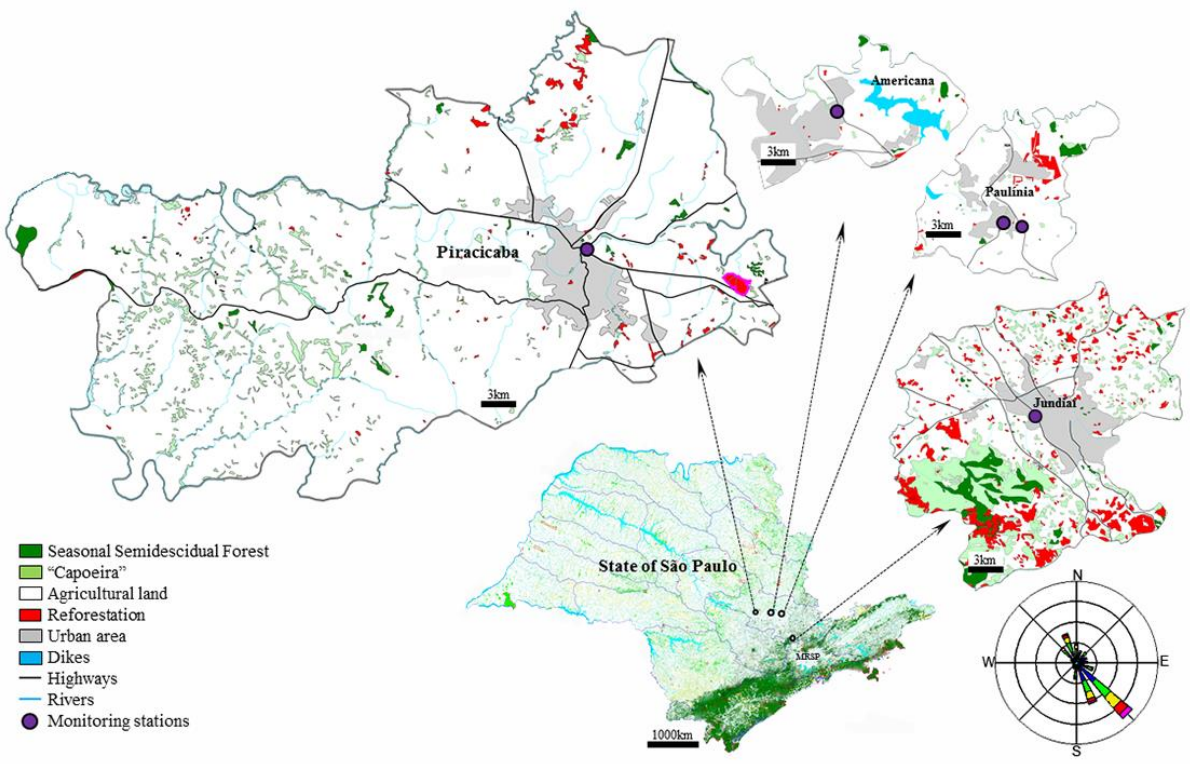

Figure 3. Location of the investigated O_3 monitoring stations (●) Maps source: Instituto Florestal/Governo do Estado de São Paulo; Wind rose source: CETESB 2006.

To predict the potential effects of O_3 on vegetation, O_3 indices were calculated. The indices were: SUM00 that is defined as the sum of all hourly concentrations in a year without a threshold; SUM60 defined as the sum of the hourly concentrations above a threshold 60 ppb in a year; SUM00 and SUM60 for six months, corresponding to dry and wet season (from April to September and October to March, respectively) and the AOT40 defined as the sum of the excess of hourly concentrations over the cut-off of 40 ppb during light hours (6:00 to 20:00) calculated over six months (April to September) as recommended by UN/ECE (2004). For this, we used data from five monitoring stations located in urban sites, downwind from the city of São Paulo, three located in the MRC region; Paulínia, Paulínia Sul and Americana, and two out of it; Jundiaí and Piracicaba (fig 3). These monitoring stations were selected in order to understate the urban localization of them and to have a better understanding of the O_3 possible effect on the local vegetation.

Jundiaí, is located 63km west from the capital São Paulo, has an area of 432km^2 and a population of approximately 370 thousand inhabitants. Its fleet is composed of approximately 130,000 light vehicles, 11,000 trucks and 25,000 motorcycles. The air monitoring station is located at 46° 53' 48" W 23° 11' 30" S in a site classified as “residential” in relation to the use of soil and exposed population, once it is located in residential neighborhoods and suburban areas. (CETESB, 2005)

Paulínia is 118km west from the capital with an area of 138.8 km^2 and a population of approximately 82 thousand inhabitants. The fleet is composed of approximately 20,000 light vehicles, 3,000 trucks and 3,500 motorcycles. The city has an expressive industrial complex, with large industries, especially in chemical and petrochemical sectors (Sousa, 2002). The air monitoring station is located at 47° 09' 14" W 22° 46' 17" S in an altitude of 751 meters classified, in relation to the use of soil and exposed population, as “commercial” once is located in a downtown area with high people and vehicles circulation. Paulínia Sul monitoring

station is located at 47° 08' 10" W 22° 47' 10" around 3,5km southeast from Paulínia monitoring station (CETESB, 2006).

Americana is located 124km west from the capital. It has an area of 134km^2 and population of approximately 210 thousand inhabitants. The air monitoring station is located at 47° 20' 21" W 22° 43' 25" S in an altitude of 545 meters, classified as commercial in relation to the use of soil and exposed population as commercial once is located downtown. The city also has a fleet of 70 thousand of light vehicles, 6 thousand heavy vehicles and 15 thousand motorcycles (CETESB, 2004).

Piracicaba is 160km from the capital. It has an area of 1353km^2, population of approximately 365 thousand inhabitants. The air monitoring station is located at 47° 38' 58" W 22° 42' 03" S in an altitude of 554 meters classified, in relation to the use of soil and exposed population as commercial once is located downtown. Piracicaba has around 1.097 industrial establishment been 57 considered as medium and high size. The city also has a fleet of 110 thousand of light vehicles, 13 thousand heavy vehicles and 26 thousand motorcycles (CETESB, 2004).

All calculations were based on quality-assured data (annual sampling efficiency ≥75%) provide by CETESB (http://www.cetesb.sp.gov.br/ar/qualidade-do-ar/32-qualar) from 2009 to 2011, for all monitoring stations excepted Paulínia's station that has data taken since 2001.

Statistical differences between the indices calculated for different stations and years/seasons were tested using ANOVA (sigmaplot 12.5). When the factor year were significant a linear regression analysis were applied.

Results

Annual and daily ozone distribution in the MRC

Considering the O_3 annual distribution, it was possible to distinguish two picks occurring along the year, the more intense in September corresponding to the beginning of wet season, and another in April corresponding to the end of the wet season (fig 4). The VDP levels were higher in September, at the begging of the wet season with values around 0.9 kPa, along the wet season the VPD decreases reaching 0.6 kPa in January and the increasing again at the end of the wet season but not reaching more than 0.8 kPa along the dry season with lower values around 0.5 and 0.8 kPa (fig 4). Although along the year average levels were not higher than 1 kPa (fig 4). The radiation increases during the wet season reaching the pick during November and December and the lowest values are registered during the dry season between May and July, also when the lower levels of O_3 occurs.

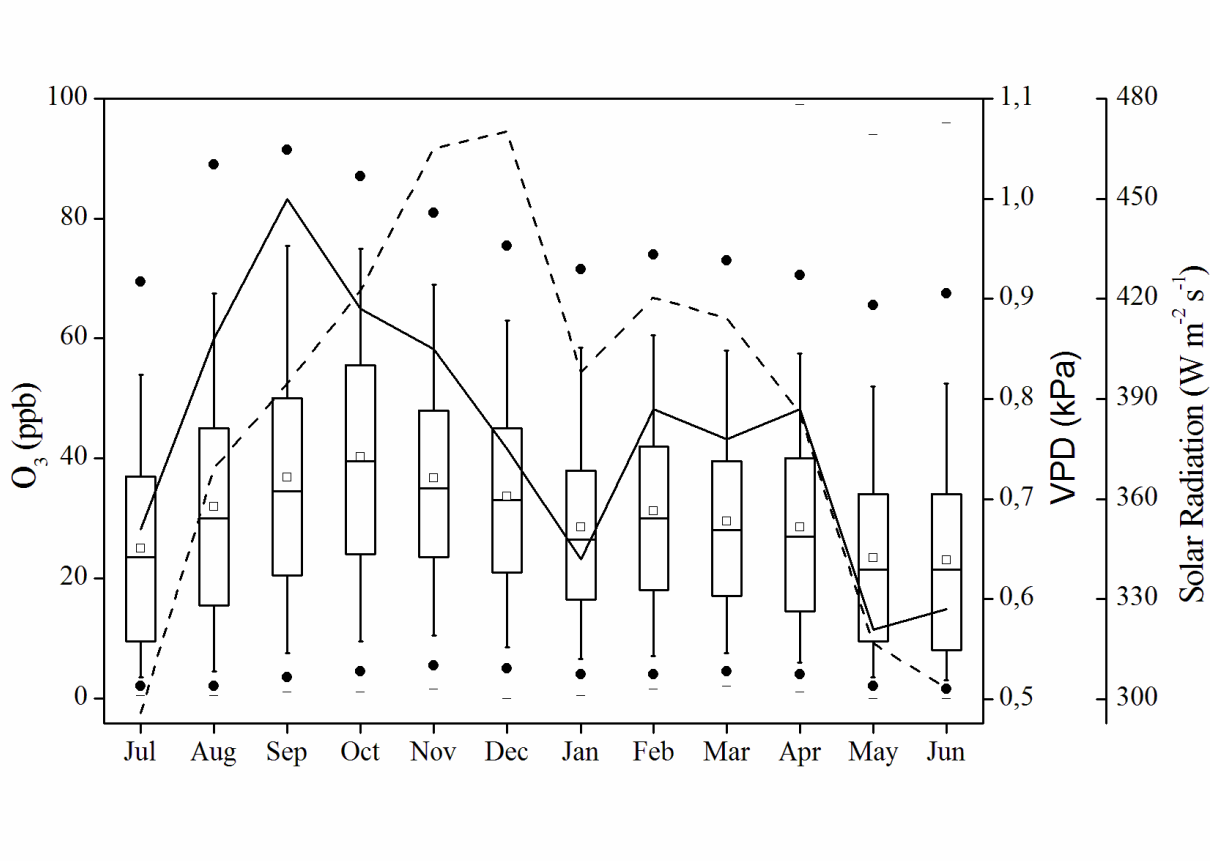

Figure 4. Annual O_3 distribution at Paulínia's monitoring station represented as a box plot: □ mean values; ● 5 or 95 percentile; ─ VPD and --- solar radiation distribution are also represented. Wet season: from October to March. Dry season: from april to September.

In Paulínia, O_3 followed a daily distribution course characteristic of urban areas (fig 5A), with the pattern of minimum values in the early morning, a significant rise during the morning with increase of solar radiation (fig 5C), occurring just after the NO and NO_2 peaks that occurs due to traffic rush hour (fig 5A and B). The highest values of O_3 were recorded in the afternoon, between 2:00 and 16:00 and then, declining during the night (fig 5A). Daily pattern of NO_2 averages (fig 5B) suggested a local photochemical production of O_3 taking place in the morning, when NO and NO_2 ratios drops and O_3 ratios starts to increase, coinciding with increasing of solar radiation.

Higher values of NO and NO_2, O_3 precursors, were detected during the dry season but daily levels of O_3 did not change substantially between both seasons, although, during the dry season, degradation took longer at night (fig 5B), once the radiation still also until later (fig 5C). Daily distribution of VPD followed the same pattern as the solar radiation and O_3 distribution, with a smooth difference between both seasons. Average values between 8:00 and 20:00 were never lower than 0.24 kPa and not higher than 1.89 kPa (fig 3C).

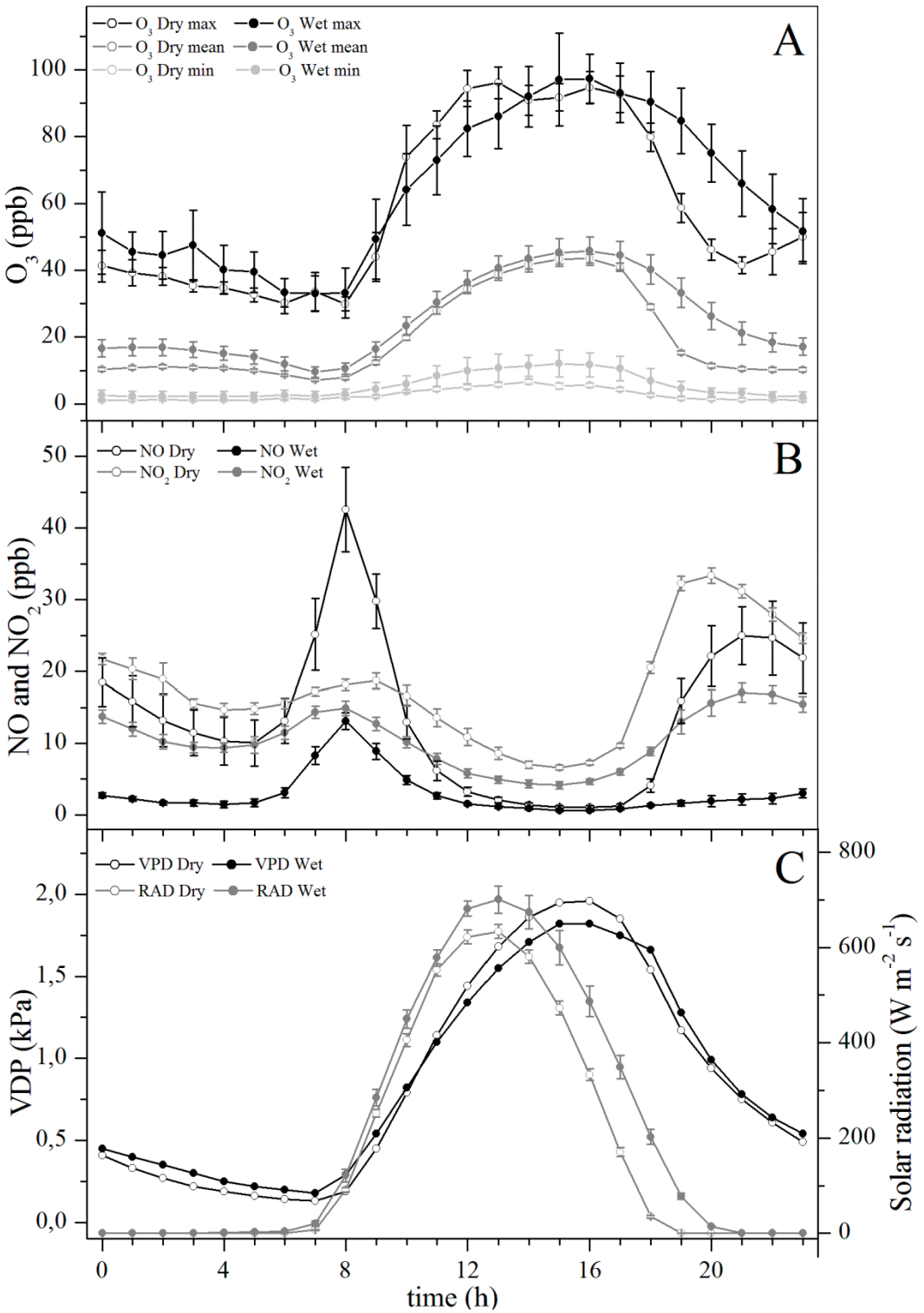

Figure 5. Averages of daily distribution parameters on Paulínia's monitoring station (data from April 2001 to March 2012): A. O_3 max, mean and min values; B. NO and NO_2; C. VPD and solar radiation. Bars = Standard error.

Ozone indices at the MRC

Paulínia's monitoring station presented the highest SUM00 index calculated annually (average of 191 ppm h.) compared to Jundiaí and Americana's stations (average of 144 and 138 ppm h. respectively). The other stations as well, no differences were found comparing the years (fig 6 A). No statistical differences between station and year were notice when considering annual SUM60.

The SUM00 and SUM60 indices calculated seasonally were significantly higher during the wet season and again, Paulinia's station presented the highest levels (SUM00 average of 112 and SUM60 average of 90 ppm h. on wet and dry seasons respectively) when compared to most of the other stations (fig). The indices SUM00 and SUM60 calculated over both, dry and wet season, during the last seasons (between October 2011 and march 2012) presented higher values comparing to the other years measured, excepted in Piracicaba. Although, considering data from all stations an increasing tendency were just confirmed for SUM60 index ($r=0,42$ $p=0,02$).

The AOT40 vegetation protection threshold set by the UN/ECE of 5 ppm for Perennials (Semi-) natural vegetation in a time period of six months (April to September) was exceeded almost every year in the five monitoring stations (fig 6E), with the highest values occurring in 2011, reaching 12.5 ppm in Paulínia station, for this index no statically differences were found between the stations or the years.

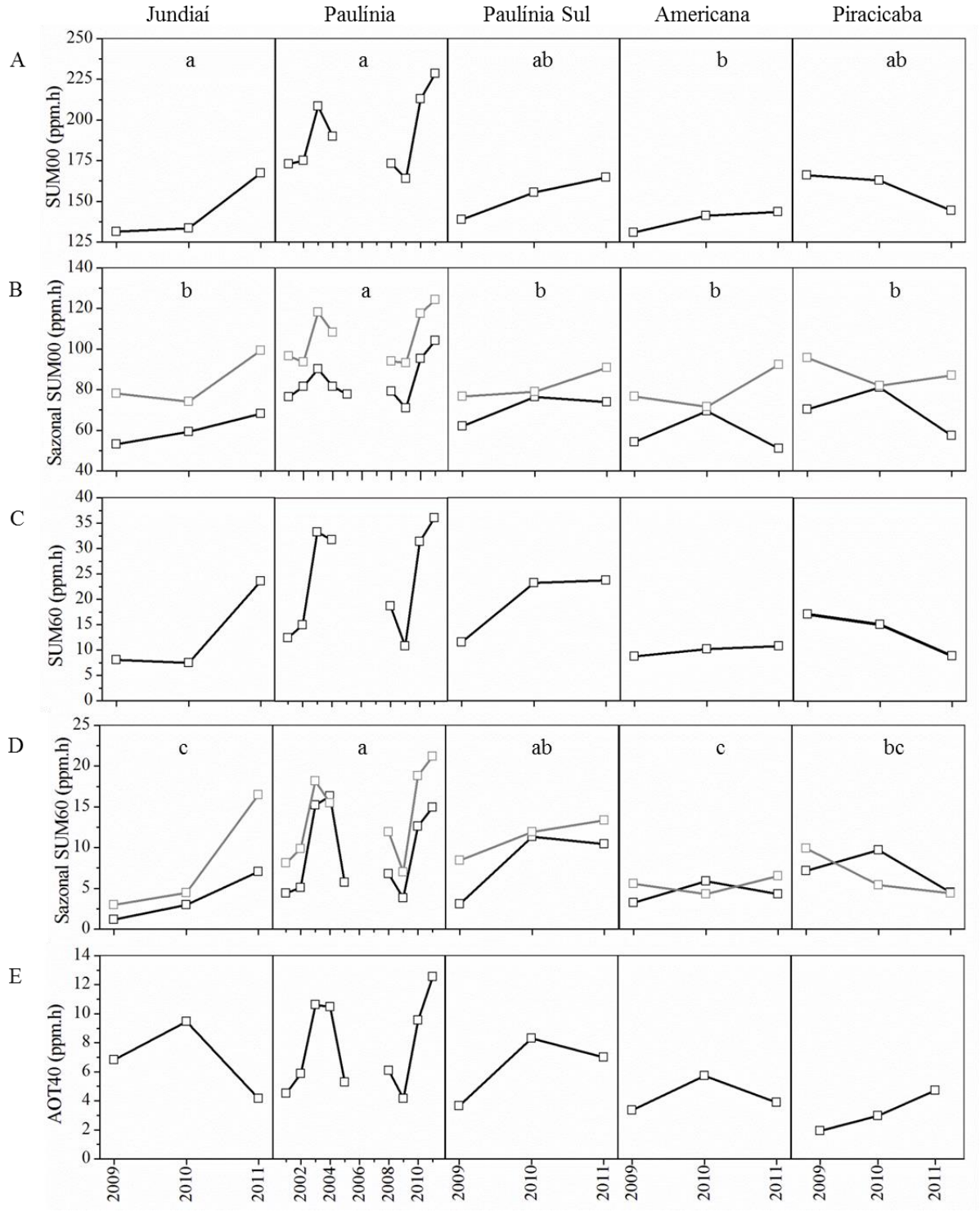

Figure 6. O_3 exposures indices on different monitoring stations, between April 2001 to March 2012 for Paulínia's monitoring station and from April 2009 to March 2012 for the other stations. Different letters on the top of each graphic means statically differences between the stations ($p < 0.05$). —□— Wet season and ─□─ Dry season.

Discussion

In the tropical climate area of MRC it is not possible to distinguish a clear O_3 season once O_3 formation occurs during the whole year. The highest value occurs at the beginning of the wet season but picks are registered also throughout the year. The high solar radiation registered along the year which average values of 388 W m^{-2} s^{-1} averages of (432 W m^{-2} s^{-1} during the wet season and 344 W m^{-2} s^{-1} during the dry season) may be the key for the constant O_3 formation in the tropical climate conditions. Even with high amounts of precipitation (average of 113 mm along the year), especially during the wet season (average of 177 mm compared with the average of 49 mm during the dry season), O_3 is formed once the rain is concentrate in late afternoon or at the begging of the evening, thus, during the morning, solar radiation is high enough to induce O_3 production.

Daily distribution of O_3 is typical of urban areas and is closed related to emission of precursors by the intense local vehicle traffic, with O_3 levels increasing right after the morning rush hours. The daily distribution of O_3 is similar to those found in other climate conditions (Bytnerowicz et al., 2008; Paoletti, 2009) but the annual distribution is completely different from temperate climate condition, where O_3 concentration can be twice as high in summer than in the winter (Castell-Balaguer et al., 2012) and a clear O_3 season can be defined.

Considering the monitoring stations evaluates, it was possible to notice that Paulínia presents higher values for O_3 index. This fact can be attributed not only due to high levels of precursors emitted by local traffic, but also by the emission from the large Paulínia's industrial park, which also contributes with O_3 precursor's emission.

It is important to notice that CETESB monitoring stations have the main purpose of monitoring air pollutants to infer about harm public health and all monitoring stations considered in this study are allocated in urban areas. Therefore, we believe that, away from the monitoring stations , near the forest fragments, O_3 indices can be even higher.

The O_3 levels are monitored in Paulínia's station since 2000 and only since 2009 in the other monitoring stations around the area. The indices variation along the years are not enough to enable inferences about long term O_3 index in MRC, but it seems some variation occurs between the years, and the year 2011 presented the highest values ever. Considering the installation of large urban-industrial centers, the expansion of roads and highways and the construction of the Paulínia refinery – REPLAN in the MRC at the beginning of the 1960's (Gutjahr, 2004) we believe this region have been suffering under O_3 stress since the implementation of these public policies in the state of São Paulo.

The average and maximum O_3 values calculated in the different monitoring stations assessed in this study, are partly comparable with values registered in regions where vegetation damage caused by oxidative stress is observed in Europe (Novak et al., 2003; Novak et al., 2005) or in the USA (Schaub et al., 2005). However, in temperate climate condition, O_3 levels are low outside the growth season (April to September) whereas in MRC the O_3 averages are still high out of this period which lead us to believe that once most the vegetation still active along the whole year, the oxidative effect of O_3 may be ever greater compared to forests from temperate region.

It is important to notice that highest ozone peaks at the MRC occurs at the beginning of the rainy season, a time when most trees flush new leaves and are actively growing, but these leaves still exposed to and continuous O_3 concentrations that can be affecting the foliage late until the dry season when many leaves may drop. However, the severity of foliage damage caused by ozone may also depend on seasonal variations in VPD, which is one of the main factors influencing stomatal conductance and thus the flux of O_3 into the plant (Gimeno et al., 1995 and Benton et al., 2000).

The temporal series of data included in figure 4 reveal that increasing concentrations of ozone observed during the rainy season coincide with the increasing VPD and also solar radiation. So, the ozone uptake and leaf injury may be less pronounced in tropical trees

exposed to these more extreme meteorological conditions, especially during spring (September to December), when maximum values of both VPD and solar radiation are registered. In contrast, the lowest values of both meteorological parameters at the beginning of the dry season increase the probability of occurring measurable damage in mature leaves, even under the chronic levels of the ozone registered in the period.

Annual indices as SUM00 and SUM60 are higher in MRC than many sites in Italy (Paolletti, 2009) and AOT40 are comparable to those registered in South-Western Europe (Gerosa et al., 2007), exceeding the European threshold, indicating O_3 toxic potential for the native vegetation of MRC.

Although the vegetation has few deciduous trees, by contrast with temperate or dry forest, the forest appearance is always green and that the dynamics of forest foliage renewal are species-specific. Souza et al. (2008) showed that some evergreen and semi-deciduous species of the sazonal semi-deciduous forest in Brazil have representative values of carbon balance features occurring on both, wet and dry seasons, thus, we believe the SUM00 and SUM60 calculated for the whole year round are the best indices to be used in tropical regions, in order to determine the O_3 toxic potential to the vegetation.

As the AOT40 is calculated from April to September, this index is not useful when we consider the O_3 effects on the tropical vegetation once it is calculated only regarding six months, and tropical vegetation dynamic is different because no growth season is clearly defined for all species.

The sensitivity of the MRC sazonal semi-deciduous forest to ozone were observed and validated by means of morphological and anatomical observation in two arboreous species, *Astronium graveolens* and *Piptadenia gonoachanta* (Anacardiaceae and Fabaceae, respectably, Moura et al, in preparing) and we believe other species may also present visual symptoms caused by O_3 oxidative stress and that this toxic air pollutant can be one more disturbance factors acting on the degradation of these important forest fragments.

Conclusion

Considering the critical levels for forest protection, O_3 levels in the MRC are high enough to cause oxidative stress on the vegetation. Although, the effect of O_3 on local forest fragments was not determinate yet, and it can be significant considering the presence of O_3 during the whole year in the region. The SUM00 and SUM60 calculated for the whole year round seems to be the better indices to be used in tropical regions. Although, attention must be taken to indices calculated considering the wet season, especially because of decidual and semidecidual species which loose its leaves during the dry season.

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Capítulo 2

Specific foliar symptoms caused by ozone stress in native trees of southern Brazil

Specific foliar symptoms caused by ozone stress in native trees of southern Brazil

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Abstract

Toxic effects of tropospheric ozone (O_3) concentrations on the natural vegetation have been primarily reported for ecosystems from the northern hemisphere. In the southern hemisphere and tropical ecosystems, the impact of O_3 is still unknown. This study has a principal objective of investigating the effects of current O_3 concentrations in three tropical tree species (*Astronium graveolens*, Anacardiaceae; *Croton floribundus*, Euphorbiaceae; *Piptadenia gonoacantha*, Fabaceae). One year old seedlings were exposed to O_3-enriched air using indoor chambers and symptomatic samples were collected on four forest fragments in the Metropolitan Region of Campinas/SP-Brazil. The microscopic symptoms have been analyzed in light and electron microscopy. During the fumigation of *A. graveolens*, visible injury was caused by reactions in apoplast in the form of massive wart-like cell wall thickenings with subsequent oxidation. Leaflets of the composite *P. gonoachanta* quickly developed stippling resulting from a hypersensitive response like (HR-like) and chlorosis. Based on a major amount of microscopical marks, the visual symptoms could be validated on samples collected in the field as O_3 reaction in *A. graveolens* and *P. gonoachanta*. However, no specific visible or microscopic symptoms were observed in *C. floribundus*. Hence, contrasting reactions were observed in the three analyzed species, suggesting a large variability of O_3 sensitivity in relation to the high biodiversity to be found in tropical environments.

Introduction

Ozone (O_3) is regarded as the air pollutant potentially most detrimental to vegetation (Matyssek and Sandermann, 2003). In Brazil pollutant concentration is higher in the southern and southeastern regions, associated with large urbanization and industrial areas (Domingos et al., 2003). The large scale industrial activities around the Metropolitan Region of Campinas (MRC), located in southeastern of the state of São Paulo - Brazil, and the transport of O_3 and its precursors from the Metropolitan Region of São Paulo (MRSP) is the main cause of high O_3 levels registered in this region (Boian and Andrade, 2012).

The O_3 levels at the MRC are considered toxic to the local semi-deciduous forest fragments (Moura, 2013, chapter 1), which are extremely fragmented and impacted, surrounded by urban, industrial and agricultural areas (Santin, 1999). In landscapes with greatest spatial forest fragmentation, O_3 exposure would have its greatest effect, since trees along the edges of the many patches would be subjected to greater deposition than trees in the interior of the patches (Kickert and Krupa, 1990).

The effects of O_3 on individual plants and the factors that modify plant response to O_3 are complex and vary with biological and physical factors such as plant species, environmental conditions, and soil moisture and nutrient conditions (Musselman et al., 2006). Monitoring O_3 symptoms in the field requires training to recognize specific visible injuries (Bussotti et al., 2003). Visible foliar injury caused by this oxidative gas has been investigated in more than 75 European and 66 North American species of native and exotic trees, shrubs and herbs, and partly validated in controlled conditions (Innes et al., 2001; Orendovici et al., 2003; Porter, 2003; http://www.gva.es/ceam/ICP-forests/).

Recent advances established that plant responses elicited by O_3 can be recognized based on markers at characteristic tissue, cell and sub-cellular locations mostly in the mesophyll (Kivimäenpää et al., 2003; Oksanen et al., 2003; Vollenweider et al., 2003; Gravano et al.,

2004; Vollenweider and Gunthardt-Goerg, 2006). Several of these marks establish a link between the intensity of sub-cellular and cellular injury and gradients of light exposure.

Evidences of effects of O_3 on forests outside Europe and the United States are very limited (Ashmore, 2005). The response of different plant species to O_3 on tropical ecosystems still significantly uncertain (Sitch et al., 2007), and its potential impact on tropical forest ecosystems needs to be specifically assessed. We have notice of only few experimental-based studies with native Brazilian species (Moraes et al., 2006; Furlan et al., 2008; Furlan et al., 2010), but the assessment of O_3 visual injury in the field with as passive monitoring has never been done and the effect of O_3 on local vegetation still unclear.

The aim of this study was to compare leaf-level macroscopical and microscopical symptoms of experimentally O_3-exposed seedlings (juvenile trees) and ambient O_3-exposed field trees (mature trees) of *Astronium graveolens* Jacq. (Anacardiaceae), *Piptadenia gonoacantha* (Mart.) Macbr. (Fabaceae) and *Croton floribundus* Spreng. (Euphorbiaceae), all three present on semi-deciduous seasonal forest fragments around the Metropolitan Region of Campinas – SP (MRC), intending to answer the following questions:

Are all three tropical species studied sensible to O_3? What are the structural markers of O_3 that trigger visible foliar injury on each species? Can we validate O_3-like visual symptoms present in the field samples by means of experiments under controlled conditions?

Material and methods

Ozone exposure - experimental approach

For experimental O_3 exposure, one year old seedlings, around 1 m high, were acquired from commercial producers (Capivari Monos - ONG). Those were transplanted into pots (20L) filled with 2/3 forest substrate and 1/3 vermiculite, fertilized with Peters (10:10:10)

once every 15 days and watered to field capacity every two or four days. The seedlings were kept in a greenhouse under filtered air for one month, and after that time, they were acclimatized inside the fumigation chamber for two weeks. O_3-enriched started on one of the chambers with plants exposure to a regime of a square wave of 70 ppb O_3, from 9 am. to 3 pm., while in the other chamber plants received only filtered air. SUM00 (sum of all hourly concentrations in a year without a threshold) was calculated to express the plants O_3 exposure. The first exposure lasted 53 days (from April 25 to June 17, 2011) and the second 36 days (from May 15 to June 05, 2012); the same procedures were used in both experiment replications.

Fumigation facility was described by Souza and Pagliuso (2009) where the O_3 was generated by an Ozontechenic™ generator and its concentration was continuously monitored with an O_3 analyzer (Ecotech™ 9810B). Climate parameters as temperature, relativity humidity (RH) and radiation (RAD) were also monitored and the vapor pressure deficit (VPD) was calculated.

Ozone exposure - visual symptoms quantification

Visual symptoms quantification were restricted to fully-expanded leaves (Table 1) presented before the beginning of the experiments. All leaves were daily exanimated with 10X hand lens to detect visible O_3 injuries. The percentage of shedding was assessed for the three species, but in compound leaves of *A. graveolens* and *P. gonoachanta,* defoliation was considered when 50% of the leaflets had fallen. Once the visible symptoms emerged on *A. graveolens* and *P. gonoachanta*, the percentage of leaves and leaflets with visual symptoms were assessed. The quantification was performed every two or four days.

The progression of the symptoms was photographed, and all evaluations were made by the same person and confirmed by a second one.

During the first experiment, samples were taken either from asymptomatic leaves from control treatment -one sample of each plant- and from symptomatic leaves of fumigated plants -one or more samples of each plant- (Table 1). On the second experiment, microscopical analysis were performed on a smaller number samples, only for comparison with the first experiment (Table 1).

Table 1. Number of plants and leaves evaluated macro and microscopically on experimental and field conditions. Light Microscopic (LM), Transmission Electronic Microscopic (TEM).

	Specie	Plants evaluated	Leaves evaluated	LM samples analysed	TEM samples analysed
Experiment					
1st	*A. graveolens*	6	27	9	4
	P. gonoachanta	6	65	11	4
	C. floribundus	6	56	9	-
2nd	*A. graveolens*	6	59	5	-
	P. gonoachanta	6	178	3	-
	C. floribundus	6	101	3	-
Field					
Americana	*A. graveolens*	7	210	2	2
	P. gonoachanta	10	900	2	2
	C. floribundus	5	450	-	-
Campinas	*A. graveolens*	10	300	6	2
	P. gonoachanta	5	450	2	2
	C. floribundus	10	900	2	-
Paulínia	*A. graveolens*	7	210	2	2
	P. gonoachanta	4	360	1	1
	C. floribundus	6	540	2	-
Cosmópolis	*A. graveolens*	10	300	8	2
	P. gonoachanta	7	630	3	3
	C. floribundus	10	900	2	-
Total experiment		**36**	**486**	**40**	**8**
Total field		**91**	**6150**	**32**	**16**
Total		**127**	**6636**	**72**	**24**

Study site, climate and ozone field levels

The evaluation of the O_3 effects in adults trees (Table 1) were conducted in Febuary/2012 in four forest fragments, located inside the MRC, in the cities of Campinas, Cosmópolis, Paulínia and Americana (Fig. 1).

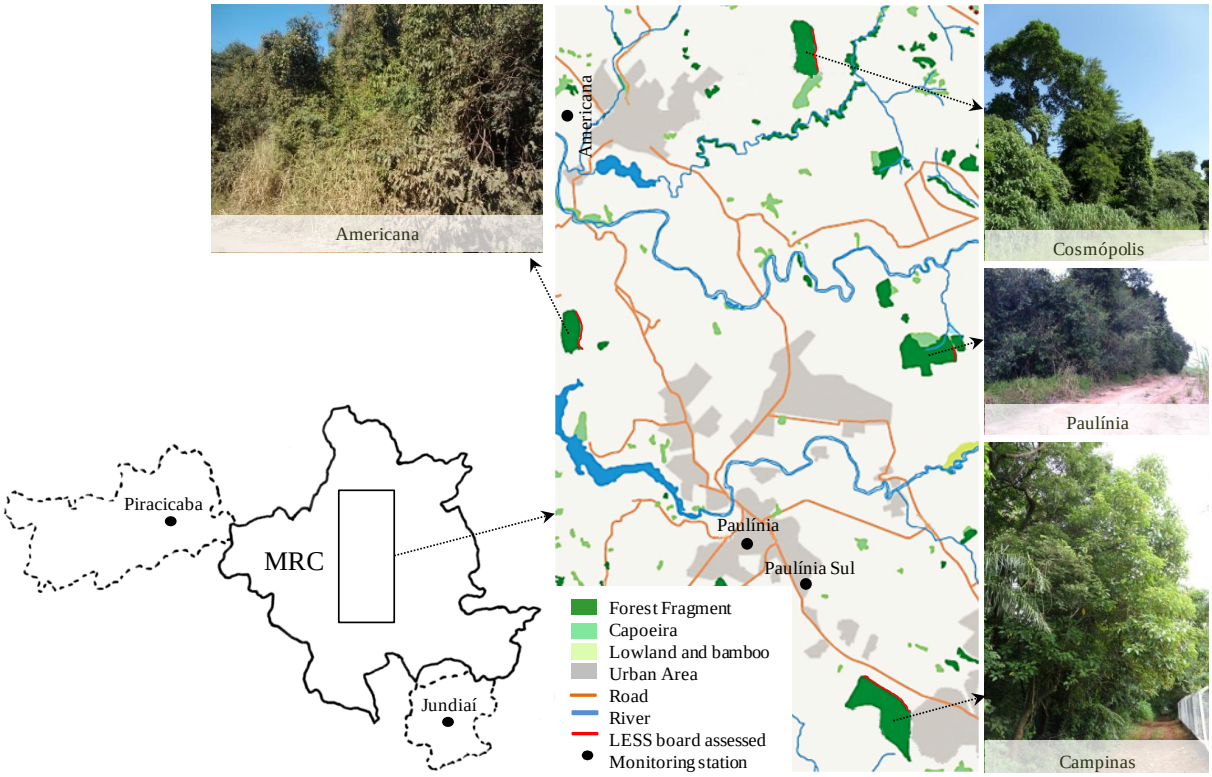

Figure 1. Forest fragments assessed in the MRC for field sampling.

To establish if the different forest fragments assessed are exposed to similar O_3 levels we calculated SUM00, SUM40 (sum of all hourly concentrations in a year above a threshold of 40 ppb in a year) and SUM60 (sum of all hourly concentrations in a year above a threshold of 60 ppb in a year). We also evaluated climate parameters as: temperature, humidity (RH), radiation (RAD) and vapor pressure deficit (VPD). All parameters were calculated based on data from 2009 to 2011, provided by monitoring stations of the Environmental Agency of São Paulo State (CETESB). Three monitoring stations inside the MRC and close to the forest fragments (Paulínia, Paulínia Sul and Americana) were used to infer about O_3 conditions at the forest fragments assessed, and two outside (Jundiaí and Piracicaba) were considered for comparisons (Fig. 1).

Identification and quantification of visual symptoms in the field

In order to standardize field sampling and provide a significant quantification of the visual symptoms, the principles advocated in the "International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests" were applied, considering specifically information available on the " Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests "(ICP, 2004). Following its suggestions the sampling was held at the forest edges exposed to light know as the light exposed sampling site -LESS- (Fig. 1). Because of the high biodiversity in the semi-deciduous seasonal forest, and consequently a low number of specimens, each forest fragment was considered as a plot.

To ensure the randomness of sampling, based on a survey of the distribution of the three species on each LESS, we calculated the overall average of individuals in each fragment (10 individuals of each species). At the fragments where the number of individuals exceeded the standard value, ten individuals were selected, on the other hand, where the value did not reach the standard, all individuals were assessed (Table 1). Three branches, with 30 leaves each for *P.gonoacantha* and *C. floribundus*, and 10 leaves for *A. graveolens* (each leaf with an average of 10 leaflets) were quantitatively evaluated, considering the percentage of plants and leaflets with visual symptoms.

Microscopical samples were performed on asymptomatic and symptomatic leaves collected on each forest fragment, taking into account the most representative samples (Table 1).

Structural observations

We evaluated control asymptomatic, fumigated symptomatic leaves sampled during the experiment and asymptomatic and symptomatic leaves sampled in the field.

Fresh hand cuts were made to evaluate the samples on specific histochemical tests and, for other structural analysis, samples with $1cm^2$ were fixed in 2.5% glutaraldehyde buffered at pH 7.0 with 0.067 M Soerensen phosphate buffer, placed under vacuum, before storing at 4°C until further processing.

Histological, cytological and histochemical observations were performed using 1.5 μm semi-thin cuttings obtained after dehydrating the fixed material with 2-methoxyethanol (three changes), ethanol, n-propanol, n-butanol (Feder and O'Brien, 1968), embedding in Technovit 7100 (Kulzer HistoTechnik) and cutting using a Supercut Reichert 2050 microtome. Material was stained and mounted either in glycerol, reagent, or DePex depending on the staining and observation technique.

Five cuts of each sample were analyzed. All sections were observed in a Leica microscope Leitz DM/RB using either diascopic and episcopic (fluorescence) light illumination depending of the stain procedure (Table 2). Micrographs were taken using either the digital Leica DC 500 camera interfaced by the Leica DC500 TWAIN software under control of the Image Access Enterprise 5 (Imagic, Glattbrugg, Switzerland) image management system (transmitted light microscopy), or the analogous micrograph system Wild MPS 48/52 using Kodak Ektachrome 400 Asa or 100 Asa films.

Table 2 - Staining methods

Stain	Reference	Solution	Staining time (min)	Color in transmitted light	Excitation (nm)	Application target	Fig
Not stained*	-	fresh cut in glicerol 50%	-	-	-	Over view	6D, E
Autofluorescence*	-	fresh cut in glicerol 50%	-	-	340-380	Chlorophyll/lignin/poly phenols	-
Toluidine blue O / p-Phenylenediamine	Feder and O'Brien, 1968 / Kivimäempää et al., 2004	1% Aq. / 1% in iso-propanol:methanol = 1:1	8	Blue / Dark gray	-	Metachromatic / Lipids	6A, 6B, 6C, 6G, 7A, 9A, 9B, 9C, 9D
PARS	Gahan, 1984	0.5% periodic acid; Schiff reagent; 0.5% potassium metabisulfite in 0.05 N HCL	10; 20; 3×5	Pink	-	Polysaccharides	6H, 6I, 7H, 7I, 7J, 9N, 9O, 9P, 11A, 11B
Coomassie Blue	Wetzel et al., 1989	0.025% comassie brilliant blue in ethanol: acetic acid 3:1	25	Light blue	-	Proteins	9H, 9I, 9J, 11C, 11D
Aniline Blue	Gerlach, 1984	0.01% in Sorensen buffer pH 8.2	10	-	340-380	Callose	7F, 7G, 9G
Calcofluor White	Munch, 1989	1% calcofluorwhite M2R in ethanol: 50%	4	-	340-380	Cellulose	7C, 7D, 7F
Coriphosphine	Weis et al., 1988	0.03% coriphosphine Aq.	2	-	450-490	Pectin	6F, 6J, 9Q, 9R, 9S
Alcian Blue	modified according to Arend et al., 2008	0.5% Alcian blue in distilled water	15	Blue	-	Mucilage	7K, 7L, 7M
Sudan Black	modified according to Gerlach, 1984	10mg Sudam Black in 5ml ethanol: 94%	Observed in reagents	Dark blue	-	Lipids	9K, 9L, 9M
Phloro-glucinol	Webster, 1979	Saturated phloro-glucinol solution in 18% HCl	Observed in reagents	Light rose	-	Lignin	-
H_2SO_4 3M	Gutmann and Feucht, 1993	H_2SO_4 3M butanol	450 W irradiation cycles of 15 s in microwave oven	Brown	-	Polymerized proanthocyanidins	9E, 9F
DMACA	modified according to Gutmann and Feucht, 1991	0.1% DMACA in butanol: 98% H_2SO_4 =20:1	As with H_2SO_4	Blue	-	Proanthocyanidins	7B
Vanilin acid*	Sarkar and Howarth, 1976	vanilin 10%	Observed in reagents	Red	-	Proanthocyanidins	-

* hand-microtome cuttings

For transmission electron microscopy, symptomatic and asymptomatic samples were post-fixed in 2% buffered osmium tetroxide (4°C) for 24h, dehydrated in ethanol, and embedded in Epon resin (M. Creuecoeur). Ultrathin 65 nm sections were cut on the same microtome used to make the semithin sections and post-stained in uranyl acetate and lead citrate. All sections were examined and photographed at Philips CM100 at the Center for Microscopy and Image Analysis (ZMB-Irchel) at the University of Zurich (UZH).

Leaf gas exchange measurements

Net photosynthetic rate (P_n), stomatal conductance to water vapour (g_{wv}) and dark respiration (R_D) were conducted on *C. floribundus* and *A. graveolens* before the beginning of the second experiment and 1, 2, 3, 4, 6, 8, 13, 18 and 28 days respectively after starting the fumigation, or until the leaf fall. Measurements were taken once a day between 9 am. and 11 am., always on fully expanded leaves of three plants maintained on fumigation chamber and on leaves of three plants from the control chamber, using a LiCor 6400 calibrated with continuous 400 ppm CO_2.

Measurements were taken at low levels of photosynthetically active radiation (PAR) (250 µmol m^{-2} s^{-1}) since it was considered the average PAR values of the experimental environment. Ten consecutive measurements were taken in each leaf, and the averages per plant and per species were calculated. The leaf average temperature during measurements was 26°C and the relative humidity was 57%.

The shedding process in *P. gonoachanta* did not allowed us to take the gas exchange measurement in this species.

Statistical analysis

Two-Way ANOVA repeated measurements were used to compare the differences between the climate parameters and O_3 indices (SUM00, SUM40 and SUM60), with the stations and years considered the factors. The same test was used to compare the parameters evaluated during both experiments as the percentage of leaves and leaflets with visual symptoms, shedding and gas exchange, with the species and the experiments (except to gas exchange) considered as factors.

Percentage of individuals and leaves with visual symptoms in the field were compared by Two-way ANOVA with the plots and the species considered as factors. For all analysis Student Newman–Keuls pos-hoc test was used to verify interactions ($p < 0.05$).

Results

Ozone exposure and climate conditions

During both experiments plants remaining the whole time in an average temperature of 26±1°C, relative humidity (RH) of 85±5%, radiation (RAD) of 300 μmol m^{-2} s^{-1} during a 8h photoperiod and VPD of 0.60 kPa. SUM00 were 24.46 and 14.80 ppm h during the first and second fumigation experiments, respectively.

In the field, the climate parameters were not statistic different between the stations and years, with year temperature average of 21.57ºC, RH of 73%, RAD of 876 μmol m^{-2} s^{-1} and VPD of 0.69 kPa.

No statistic significant differences were found considering SUM40 and SUM60 indices even between different monitoring stations or different years. SUM40 and SUM60 averages were 57.03 and 15.88 ppm h respectively. Also, no difference were found for the SUM00

between the years, but a statistic significant difference were found between the monitoring stations being the indices higher in Paulínia (196.31 ppm h), Paulínia Sul (151.07 ppm h) and Piracicaba (161.28 ppm h) than in Americana (132.48 ppm h) and Jundiaí (137.73 ppm h).

Visual symptoms - characterization and quantification

In response to O_3 fumigation, *A.graveolens* leaves developed intercostal stippling visible on both leaf sides (Fig. 3A-H); the percentage of symptomatic foliage increased quickly and after 10 days of exposure 60% of the leaves developed visual symptoms in more than 50% of the leaflets (Fig. 2B-C). Shedding occurred continually, reaching more than 60% at the end of the experiment (Fig. 2A). In the field, visible symptoms showing morphological traits similar to those found in fumigated samples (Fig. 3E-H) were observed in 39.4% of the trees and 5.8% of the leaflets analyzed.

During the fumigation experiment *P. gonoachanta* leaves developed small brownish mottle on the adaxial surface (Fig. 3I-O), occurring in up to 90 % of the leaves and leaflets after only 20 day of exposure (Fig. 2B-C). Leaves shedding were extremely quickly and after less than 10 day of exposure 40% of the leaflets had fallen, and after 40 days none leaflets were present anymore (Fig. 2A). Visible symptoms, similar to those detected during fumigation, were found in 85% of the individual evaluated in the field occurring in an average of 4.2% of the leaflet.

Leaves of *C. floribundus* fumigated with O_3 and sampled in the field developed no specific visible ozone-like injury (Fig. 3P-T), but the former showed accelerated senescence and premature leaf shedding (Fig. 2A).

Regarding the differences between the fumigation experiments, shedding was significantly higher during the second experiment, and in both experiments, the percentage of

shedding was significantly higher on *P. gonoachanta*, while no differences occurred between *A. graveolens* and *C. floribundus* (Fig. 2A-B).

Taking into account the percentage of symptomatic leaves and leaflets in *A. graveolens* there were no significant differences between both experiments while, *P. gonoachanta* presented higher percentage of symptomatic leaves and leaflets during the first experiment (Fig. 2C-F). A significant difference between species occurred only on the second experiment when *A. graveolens* presented more symptomatic leaves and leaflets comparing to *P. gonoacantha* (Fig. 2C-F).

Considering quantification made in the field samples, no statistic difference were found between the percentage of symptomatic individual and symptomatic leaflets between plots and species, although we believe that the results of *P. gonoachanta* may be underestimated, due to the extremely capacity of this species in shedding its symptomatic leaves, as shown in the fumigation experiment in which the shedding was very fast and intense.

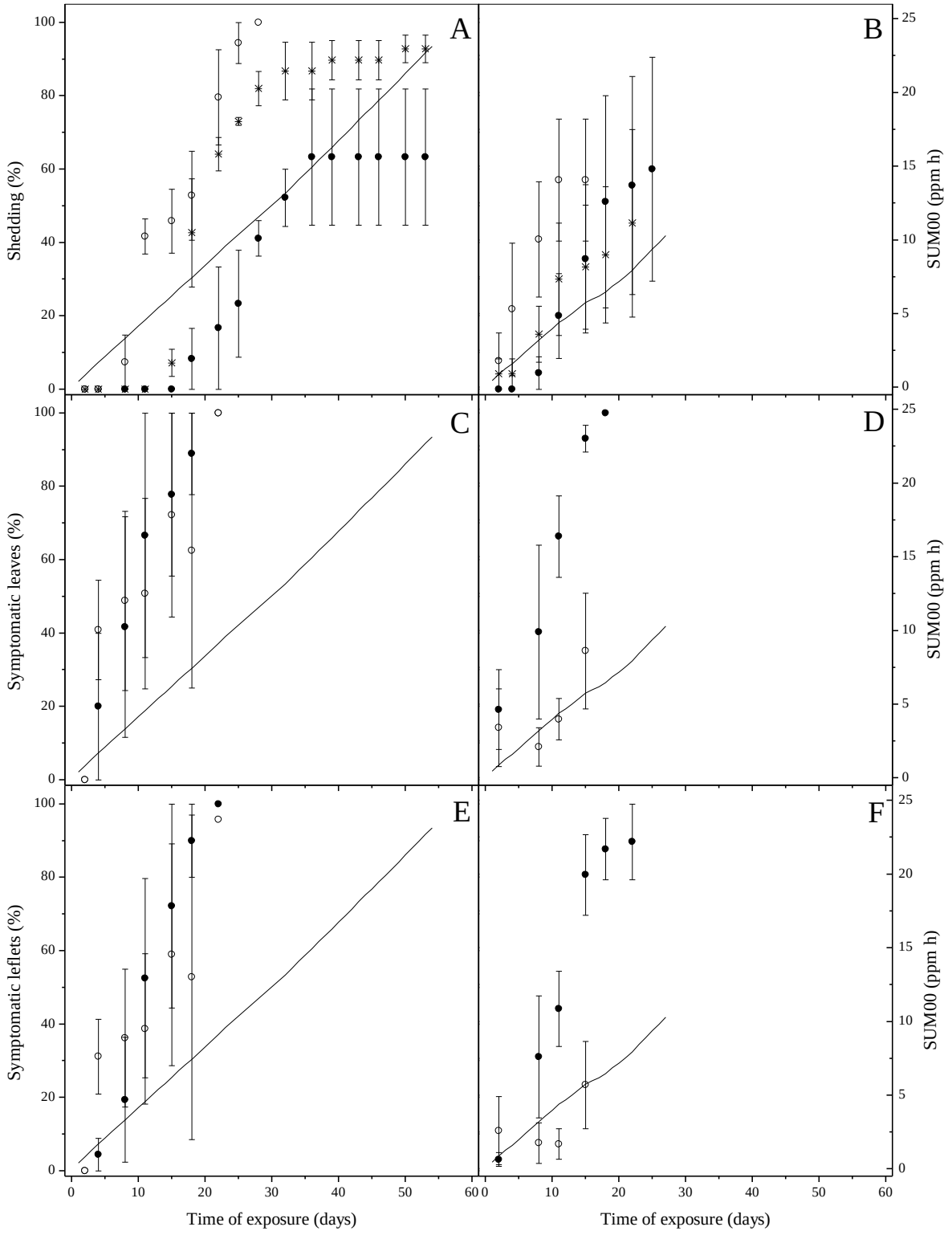

Figure 2. Visible symptoms quantification carried out during the fumigation experiments. A. Shed foliage percentage, B. Percentage of symptomatic leaves and C. Percentage of symptomatic leaflet. ● *A. graveolens*; ○ *P. gonoachanta*; * *C. floribundus*; — SUM00. A, C and D = first experiment. B, D and F = second experiment.

Figure 3. Different visual symptom expression. A-H. *A. graveolens*. A. Overview of seedling used in the fumigation experiment (control), B. Overview of tree samples in the field (leaves on detail), C. Leaflet without visual symptom, D. Visual symptoms from fumigated material, E. Visual symptom assessed in the field sampling, F. Detail of C, G. Detail of D, H. Detail of E. I-O. *P. gonoachanta*. I. Overview of seedling used in the fumigation experiment (control), J. Overview of tree sampled in the field, K. Visual symptoms from fumigated material, L. Visual symptom assessed in the field sampling, M. Leaflet without visual symptom, N. Detail of K, O. Detail of L. P-T. *C. floribundus*. P. Overview of seedling from the fumigation experiment (control), Q. Overview of tree sampled on the field, R. Leaflet without visual symptom, S. Chlorotic leaf of fumigated material, E. Chlorotic leaf of field sample.

Structural changes

The summary of the oxidative markers of each species are present on Figs. 4 and 5.

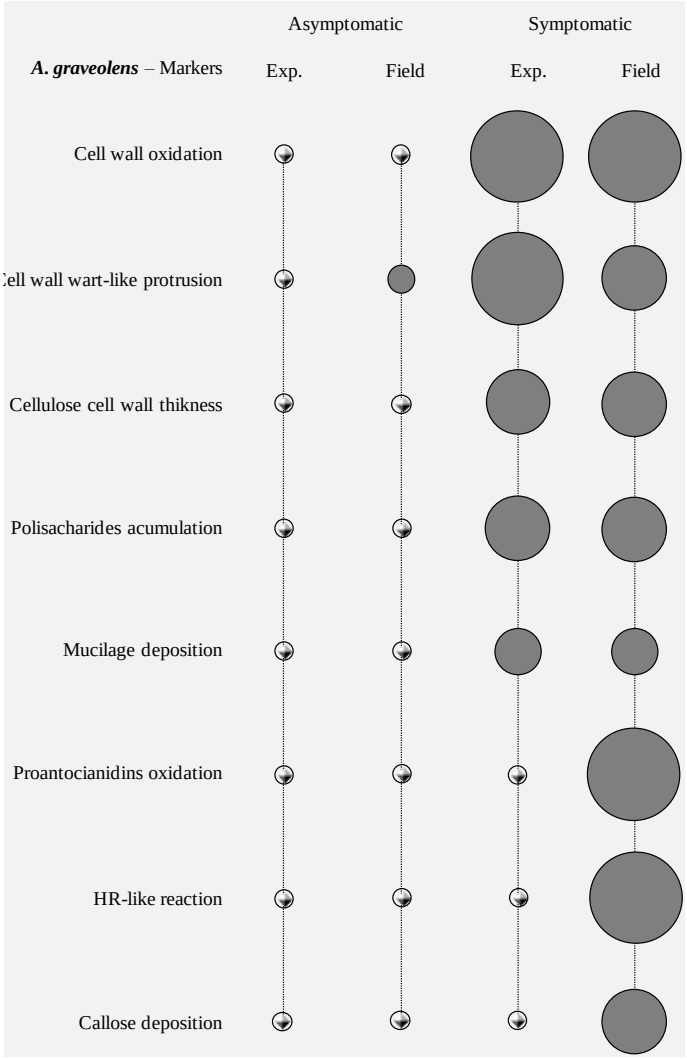

Figure 4. Main important microscopical markers of O_3 oxidative stress on *A. graveolens*. As bigger are the balls as intense it is the marker, clarifying which are the best markers to be used for O_3 symptom validation. ◒= marker not present.

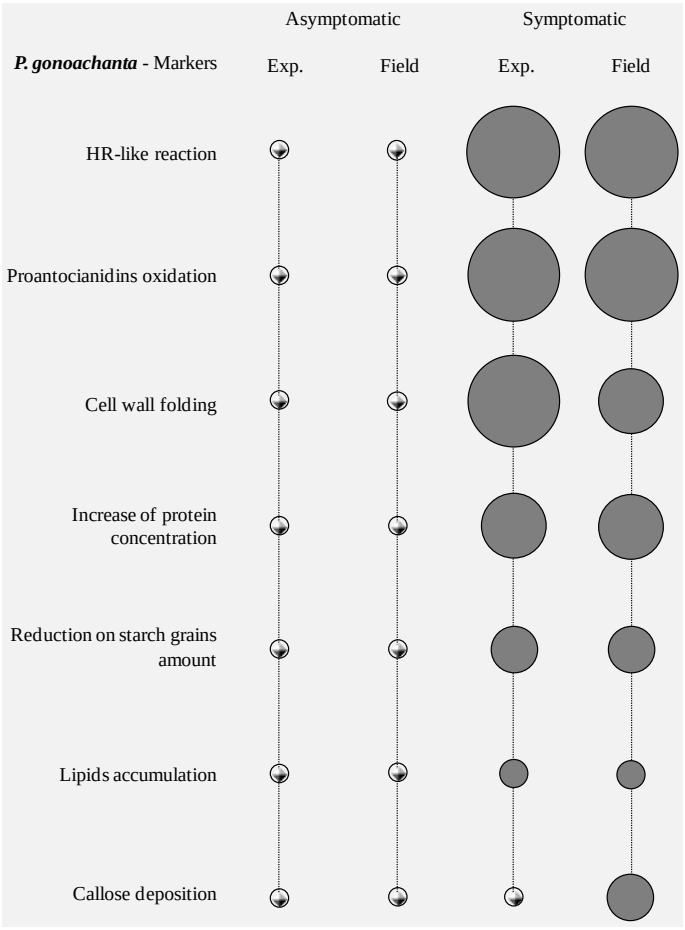

Figure 5. Main important microscopical markers of O_3 oxidative stress on *P. gonoachanta*. As bigger are the balls as intense it is the marker, clarifying which are the best markers to be used for O_3 symptom validation. ⊖= marker not present.

In *A. graveolens* symptomatic leaves either from O_3 fumigated plants or sampled in the field, visible symptoms were related to an extreme oxidative reaction (Fig. 6D-E), although the structural changes on both samples were only partially similar. On fumigated samples we observed numerous massive wart-like thickenings on cell wall protruding in the apoplast, mostly on spongy parenchyma cells near the substomatal chamber (Fig. 6G versus Fig. 6A). These protrusion were composed by polysaccharides (Fig. 6H), principally pectin (Fig. 6J) and were the most prominent structural change observed in this species. Furthermore, the protrusions, suffering oxidation in an abaxial to adaxial gradient (Fig. 6H), were responsible for the brownish of the visible injury. Wart-like pectic protrusions were also detected on

symptomatic field samples (Fig. 6F and Fig. 6I), but they were not present on asymptomatic field samples (Fig. 6B) or on samples from the control experimental chamber (Fig. 6C).

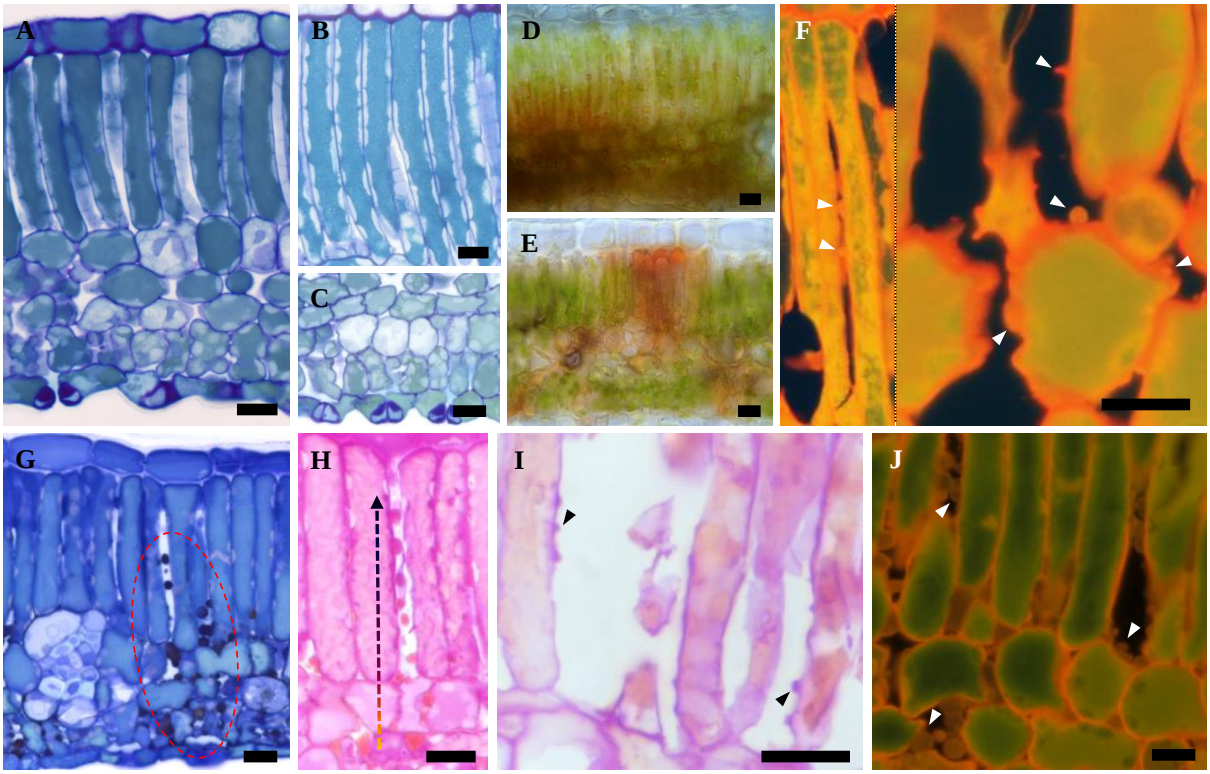

Figure 6. Histological markers for O_3 stress in leaves of *A. graveolens*. A-C asymptomatic samples. A and C sample from experiment. B. sample from field. D, G, H and J symptomatic samples from experiment. E, F and I symptomatic sample from field. On experimental and field conditions the oxidative burst was intense (D and E). Polysaccharides cell wall wart-like protrusions (arrowhead) occurred on fumigated and field samples (H and I, respectively) composed of pectin (J and F, respectively). On fumigated samples it is possible to notice the protrusion formation, concentrated on the spongy parenchyma (G) with its oxidation occurring from abaxial to adaxial direction (H, arrow). Bars = 10 µm.

The formation of wart-like protrusions in the field samples was less massive than in the fumigated samples. Moreover, the visible symptom in the field samples were caused mainly by the substantial gradient of oxidized tannins (Fig. 7B), present on the palisade parenchyma cells, more intense in the upper cell portion. This tissue was the most severed injured, presenting a HR-like reaction (Fig. 7A), identified by the quick collapse of the palisade parenchyma, occurring in distinct cells, where organelles such as chloroplast still distinguishable, but drastically disrupted (Fig. 8B-C versus Fig. 8A).

Cellulose cell wall thickness (Fig. 7D-E versus Fig. 7C) was observed especially on the spongy parenchyma cells of either fumigated and field symptomatic samples; in the field samples, callose deposition was also present on the cell walls of the palisade parenchyma cells (Fig. 7G versus Fig. 7F). On both cases, an accumulation of polysaccharides were observed surrounding the vacuolar tannins of palisade cells (Fig. 7I-J versus Fig. 7H) and a mucilage deposition between the cell wall and the vacuole (Fig. 7L-M versus Fig. 7K) also occurred. These accumulations were not present on asymptomatic samples.

In the field symptomatic samples, the palisade cells without HR-like reaction presented an increase of the number of plastoglobules when compared to asymptomatic samples (Fig. 8G versus Fig 8E). This phenomenon also occurred on fumigated symptomatic samples comparing to control experimental samples (Fig. 8F versus Fig. 8D).

The higher number of plastoglobules also occurred on the chloroplast of spongy parenchyma cells of symptomatic field and fumigated samples (Fig. 8J-K versus Fig. 8H-I). Less and smaller chloroplasts were observed on spongy cells of symptomatic samples (fumigated and field), and the grana arrangement were not as well defined as they were in the asymptomatic samples (Fig. 8J versus Fig. 8H).

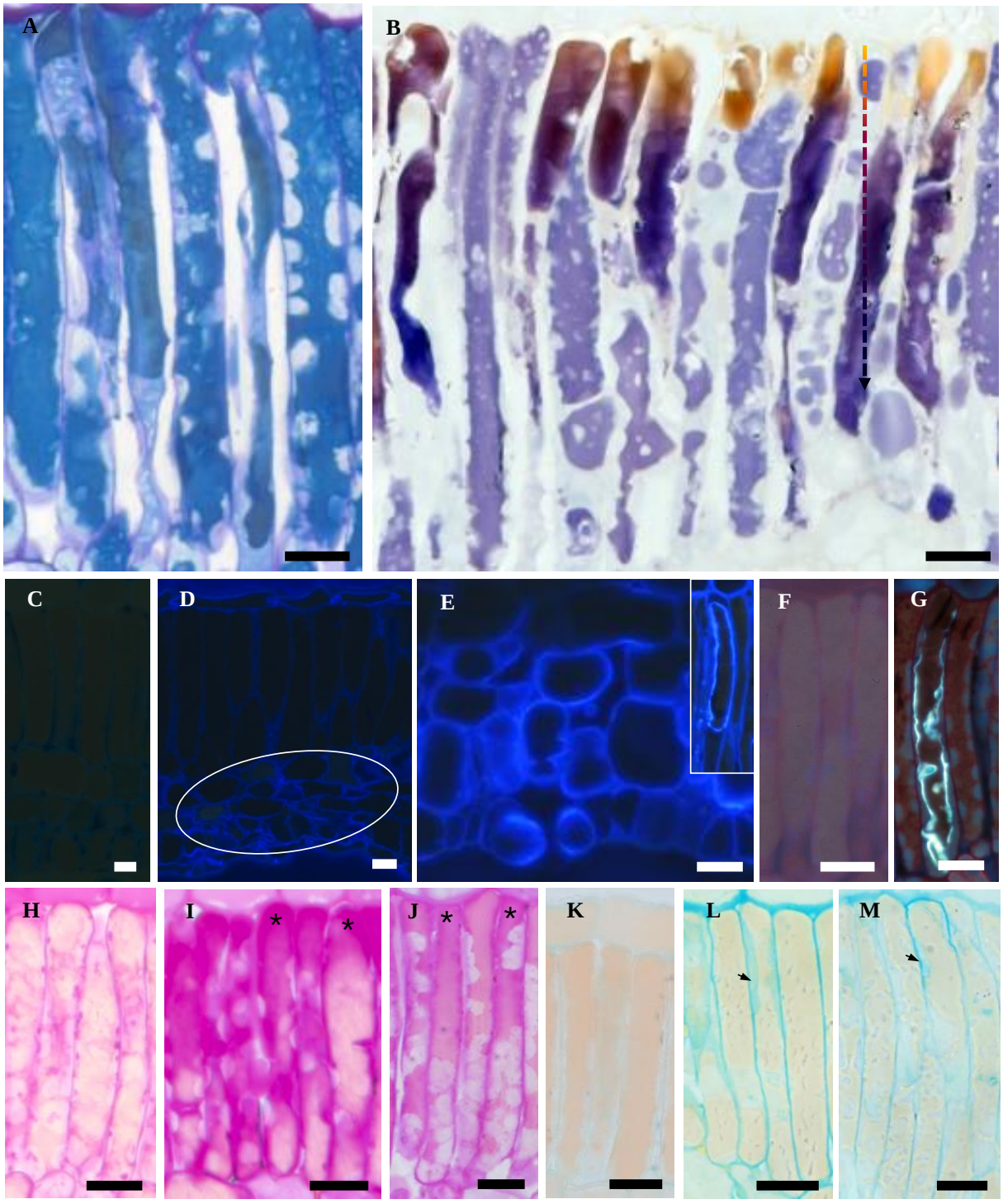

Figure 7. Histological markers for O_3 stress in leaves of *A. graveolens*. C, F, H and K from asymptomatic samples. C and H from experiment. F and K from field. D, I and L symptomatic sample from experiment. A, B, E, G, J and M symptomatic sample from field. A. HR-like reaction occurring on the palisade parenchyma cells. B. Gradient of tannins oxidation inside HR-like cells (dot arrow). D and E. Cellulose deposition on spongy parenchyma cells, and palisade HR-like cells (E detail above). G. Callose deposition on HR-like cells. I and J. Gradient of polysaccharides deposition on palisade parenchyma cells (*). L and M. Mucilage deposition on palisade parenchyma cells (arrow). Bars = 10 µm.

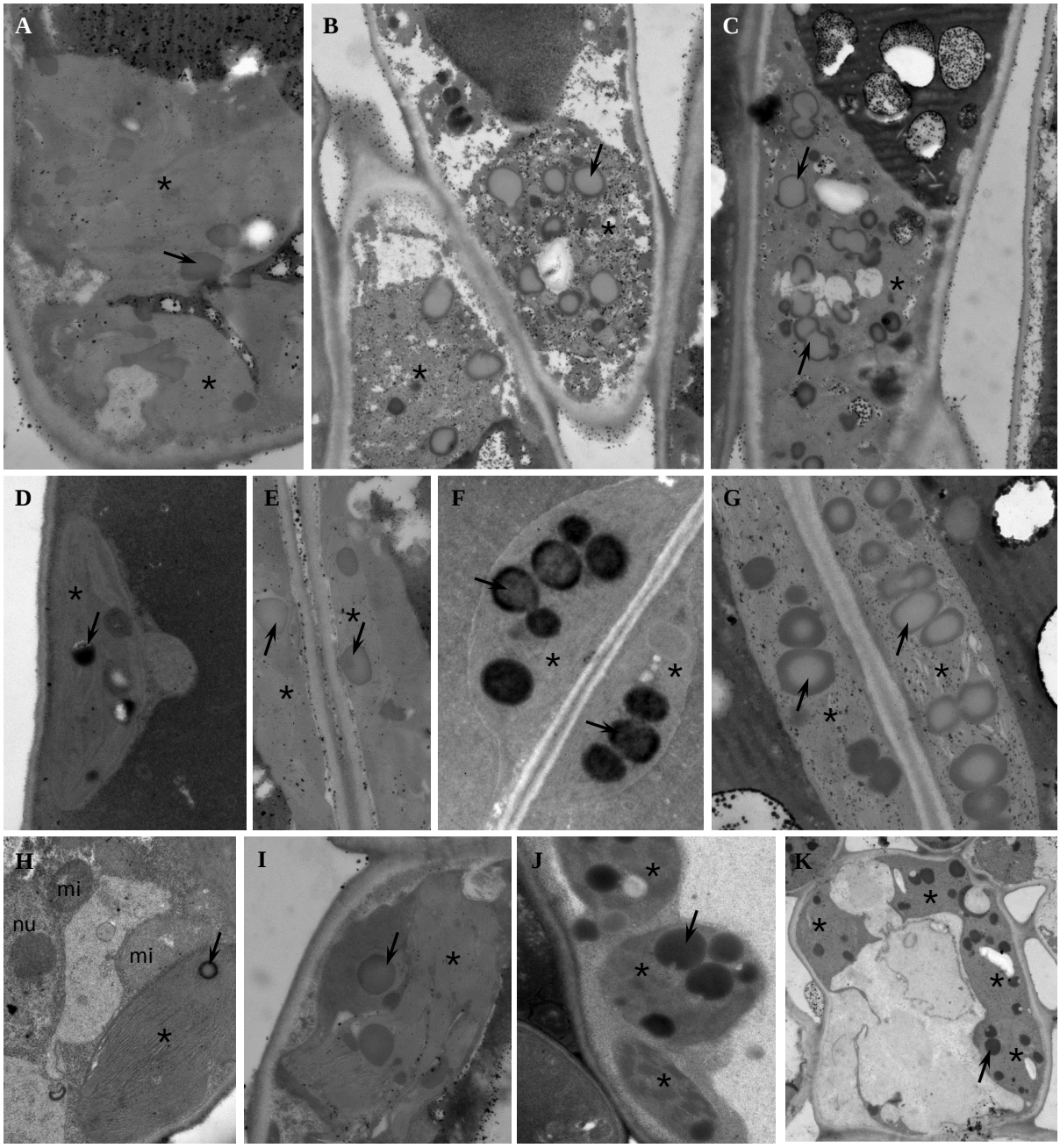

Figure 8. Cell markers for O_3 stress in leaves of *A. graveolens*. A, D, E, H and I from asymptomatic samples. A, E and I from field. D and H from experiment. B, C, F, G, J and K from symptomatic samples. B, C, G and K from field. F and J from experiment. On HR-like cells (B and C) the chloroplasts (asterisk) are completed disrupted and it is not possible to distinguished the granna and tilacoids structure. On symptomatic samples a increasing of plastoglobules (arrow) occurred even on palisade (F and G) or spongy parenchyma cells (K and J). Spongy parenchyma cells of asymptomactic samples from field are not as healthy (I) than on control samples from the experiment (H). Figures magnifications: E = 33000X; A, G, H, I = 24000X; D, F, J = 17500X; B, C = 9700X; K = 7400X.

Either, on fumigated or field symptomatic leaflets of *P. gonoachanta*, the mottles were produced by a strong HR-like reaction affecting exclusively restricted groups of palisade parenchyma cells (Fig. 9C-D versus Fig. 9A-B), presenting the following histological and cytological markers: (1) proantocianidins oxidation (Fig. 9E-F), (2) increase of protein concentration (Fig. 9I-J versus Fig. 9H), (3) cell wall folding (Fig. 10E-F), (4) less amount of plastoglobules inside disrupted choloplast (Fig. 10C-D versus Fig. 10A-B), (5) pectin cell wall protrusions on spongy parenchyma cell wall (Fig. 9R-S versus Fig. 9Q), (6) reduction on starch grains amount (Fig. 9O-P versus Fig. 9N), (7) chromatin condensation (Fig. 10C versus Fig. 10H and Fig. 10G). Only in the field samples callose deposition occurred on the upper portion of palisade HR cells (Fig. 9G) and lipids accumulation did not occur only on control chamber samples (Fig. 9K-M).

On spongy parenchyma cells an increased number of plastoglobules (Fig. 10J-K versus Fig. 10I) inside not disrupted chloroplasts were noted on samples from both fumigated and field.

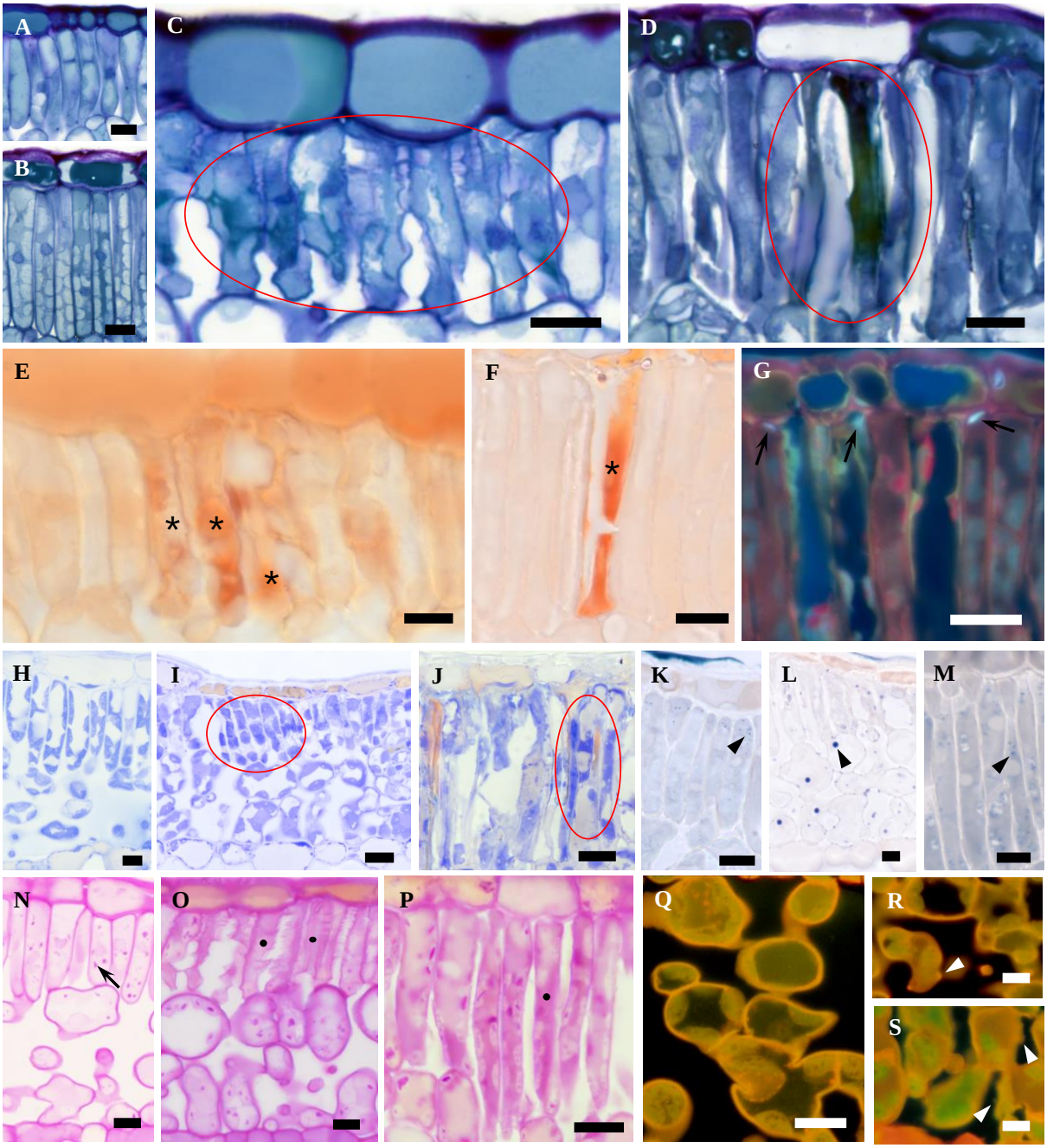

Figure 9. Histological markers for O_3 stress in leaves of *P. gonoachanta*. A, B, H, K, N and Q from asymptomatic samples. A, H, N and Q from experiment. B, and K from field. C, D, E, F, G, I, J, L, M, O, P, R and S from symptomatic samples. C, E, I, L, O and R from experiment. D, F, G, J, M, P and S from field. C and D show palisade parenchyma cells with HR-like reaction compared with healthy cells in A and B. The proantocianidin oxidation occurs in HR-like cells (E and F) where a callose deposition is noted in the field sample (G). Protein from disrupted chloroplasts accumulated within the cells (I and J - red ellipse) compared to proteins inside healthy chloroplasts located at the cells edge (H). K, L and M show lipids accumulation on mesophyll cells (black arrow head) not directed related to the HR-like process. On HR-like cells no starch grains is accumulated (O and P dots) comparing to control samples where the starches are storage inside the chloroplasts (N). Wart-like protrusions (R and S white arrow head) were found all symptomatic samples but no on control ones (Q). Bars = 10 µm.

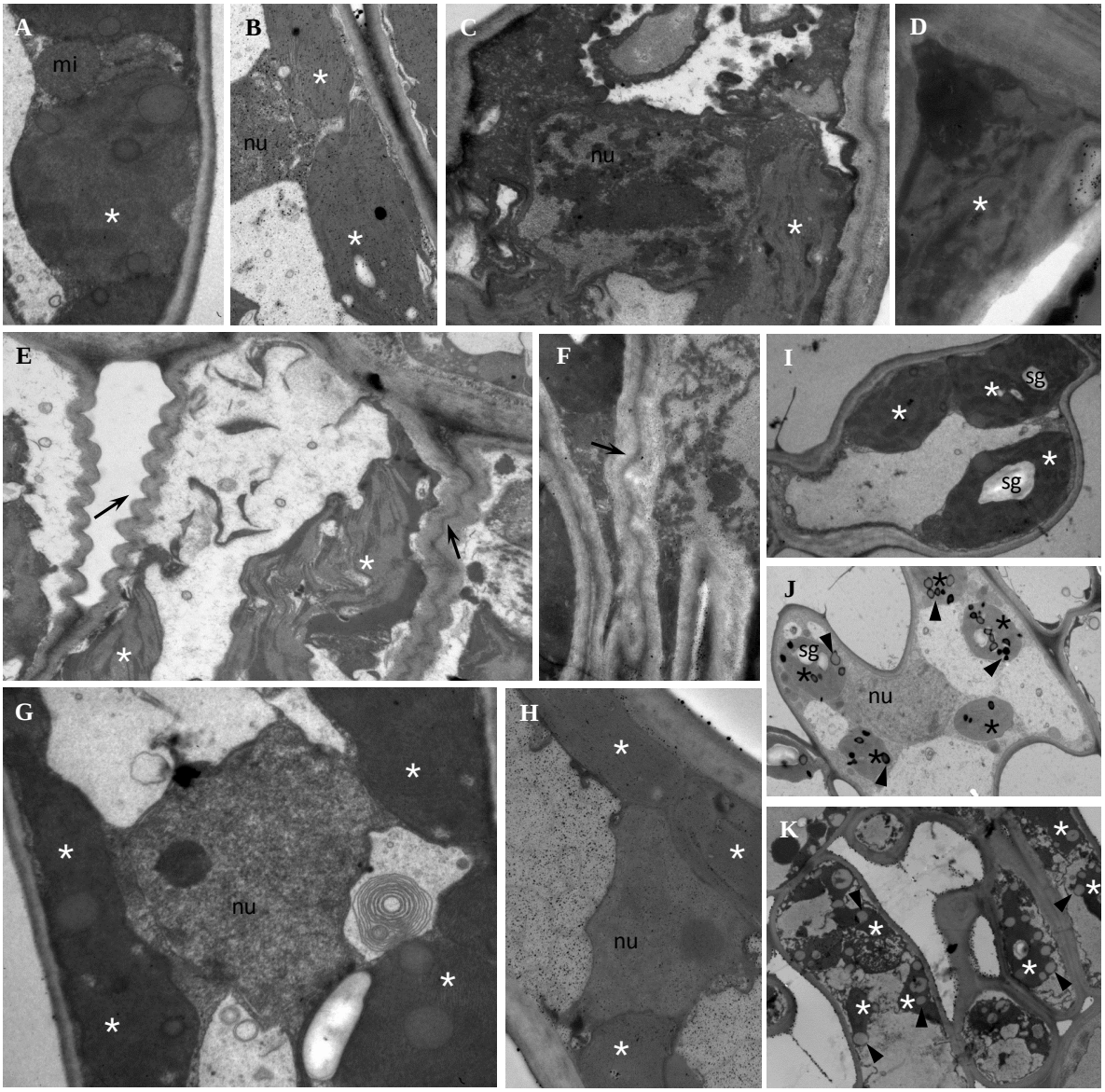

Figure 10. Cell markers for O_3 stress in leaves of *P. gonochanta*. A, B, G, H and I from asymptomatic samples. B and H from field. A, I and G from experiment. C, D, E, F, J and K from symptomatic samples. D, F and K from field. C, E and J from experiment. On HR-like cells (C and D) the chloroplasts (asterisk) are completed disrupted with an indistinguishable structure (D) and the nucleus presents chromatin condensation (C). On healthy cells chloroplast structure is well distinguishable (A and B) and the nucleus is active with apparent nucleolus (G and H). Cell wall folding (E and F) occurring on HR-like cells (arrows). Accumulation of plastoglobules (arrowhead) inside spongy parenchyma cells (J and K) and healthy chloroplasts with starch grains (sg) and well define grana (I). Figures magnifications: B, C, D, F, H = 24500X; E = 17500X; A, G = 13500X; I = 7400X; J = 5800X; K = 4200X.

No specific structural markers were found either on fumigated or field samples of *C. floribundus*, only indication of senesce process marked by no starch grains accumulation

(Fig11. A versus Fig. 11B) and the loss of the protein content inside, a less amount of rounded chloroplasts with many plastoglobules (Fig. 11D versus Fig. 11C).

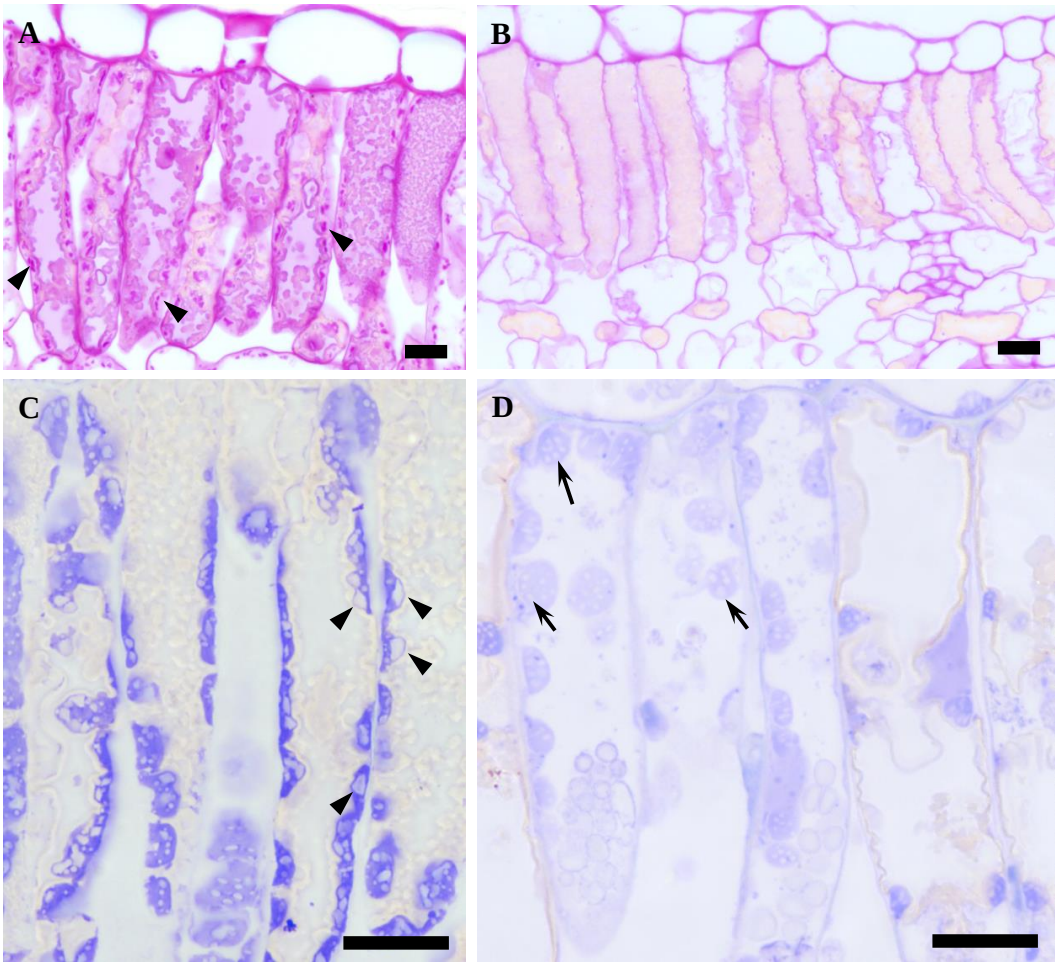

Figure 11. Histological markers of senescence in leaves of *C. floribundus*. A and C from asymptomatic experiment samples. B and D from symptomatic experiment samples. Samples in advanced chlorotic stage do not accumulate starch grains (head arrows) inside de chloroplasts (B versus A). Chlorotic samples also have less chloroplasts with many plastoglobules (arrows) and a weak reaction to protein stain (D versus C). Bars = 10 µm.

Gas exchange

Comparing control plants of both species, we observed higher gas exchange values in *C floribundus* than in *A. graveolens*, which indicates its status as a pioneer species. On average (over all measurements) *C. floribundus* makes 61% higher P_n and 35% higher g_{wv} than *A. graveolens*.

Ozone clearly reduced gas exchange in fumigated leaves of *A. graveolens* along the experiment, with significant differences in P_n and g_{wv} after two days of exposure After 28 days of the experiment, P_n decreased by 54% and g_{wv} by 63% in comparing with control plants (Fig. 12A-B). Moreover, R_D rate significantly increased in plants grown under O_3 condition (Fig. 12C).

C. floribundus presented no statistically significant differences between the two treatments for P_n and g_{wv} (Fig. 12 A-B) but the R_D was significant higher in plants fumigated along the experiment period (Fig. 12C).

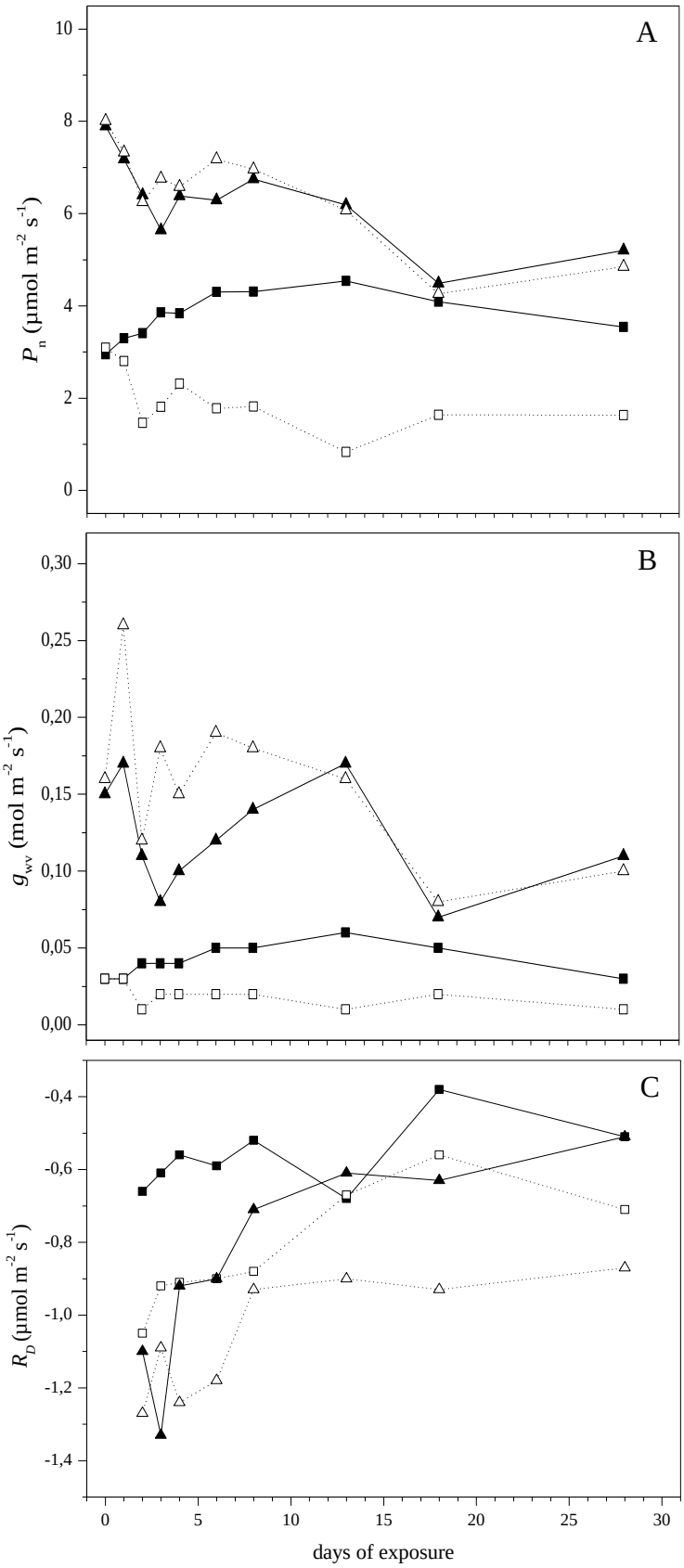

Figure 12. Treatment effect for: A. Net photosynthesis (P_n), B Stomatal conductance (g_{wv}). C. Dark respiration (R_D). *A. graveolens* (■ control; □ fumigated). *C. floribundus* (▲ control; Δ fumigated).

Discussion

The toxic effect of O_3 on tropical trees

Within a universe of over 450 species identified only in Campinas city by Santin (1999) only three species were investigate in this study; nevertheless, the structural diversity of these species, as well as differences in responses to exposure to O_3 is surprising.

Visual symptoms were found on every plot either in *A. graveolens* and *P. gonoachanta* showing us that the injuries caused by O_3 are homogeneous distributed in the forest fragment assessed. Even with few differences on the O_3 SUM00 index at the different monitoring stations around this fragments, the species studied has been exposure to toxic levels of O_3 (Moura, 2013, chapter 1). The SUM00 indices calculated in the MRC are comparable to the indices which causing negative effects to the vegetation and to human health in Italy (Paoletti et al., 2007), and we believe this negative effect is also occurring at the MRC; moreover, it is important to take into account that parameters as the vapor pressure deficit (VPD) favors the O_3 uptake along the whole year in the MRC (Moura, 2013, chapter 1) which let us to believe that the O_3 affect in the MRC may be even stronger that in the Europe.

Phenological researches with the studied species describe *P. gonoachanta* as a deciduous species (Gandolfi et al., 2009), which loses its leaves during the dry season. On the other hand, even knowing that *C. floribundus* also lose mostly of its leaves during the dry season, Ferraz et al. (1999) showed that new leaves are continuing flushing along the year as a strategy to maintain the photosynthetic activity. Considering this information and the observation made in the field, we believe the *P. gonoachata* and *C. floribundus* leaves evaluated in the field flushed during the beginning of the wet season in September/2011, being exposed to O_3 for almost 5 months, until in January/2012 when the sampling took place.

There is a relationship between leaves abscission and restrictions imposed by soil and atmospheric drought, although, Gutiérrez-Soto et al., (2008) showed that growth of *A. graveolens* in Cañas, Costa Rica, does not stop during the dry season, with gas exchange activity been substantial in leaves of different ages during most of the year, even in older leaves. According to the authors, a high xylem water transport capacity is maintained and intermittent leaf production occurs also during the dry season. Based on this information and on the observation made in field, it was possible to recognize the differences between new branches from the old ones. Older leaves are darker green with a thick aspect and it was on those that the oxidative stress symptoms were found. We believe the visual symptoms occurred in the field only on older leaves, which had been exposed to O_3 for around 18 months.

Much of the knowledge of the effects of O_3 on plants is derived from controlled environment or field chamber exposure studies that provide us a basic understanding of cause and effect relationships, although, results from such studies cannot be directly extrapolated to the chamberless ambient environment (Kupra, 2001). However, O_3 is probably the stress factor for which microscopical validation has been most successfully applied (Günthardt-Goerg and Vollenweider, 2007).

Paoletti et al. (2009) have shown that structural and morphological markers O_3 effect can be different depending on the O_3 concentrations in the air, the entrance of this gas inside the leaves (ozone uptake) and light conditions, indicating that plasticity in plant responses on experimental or field samples are expected.

According to Vollenweider et al. (2003), no marker taken alone is appropriated to be specific to O_3. Therefore, by combining all the indications, the observer may differentiate between the effects of O_3 and those of other stress agents. In the present study the radiation was the most different parameter between experimental and field conditions, but based on several markers the symptom validation was possible to be done on two of the three species

tested. Besides, a relation between the increasing SUM00 and the development of the visual symptoms on *A. graveolens* during the experiment confirms it sensitivity and potential as bioindicator species.

O_3 marks and validation

A.graveolens

The visual symptoms of *A. graveolens* are easily recognized in the field, compared to other type of symptoms not related to oxidative stress (Annex 1) and are similar to those developed during the experiment, probably because both are due to the oxidative process that causes a brownish stippling under the veins.

On both, experimental and field symptomatic samples, an intensive apoplastic reaction took place and on fumigated samples, this was the most important marker that trigged the development of the visual symptom The O_3 enters the leaves via stomata reacting on the apoplast, where reactive oxygen species (ROS) are generated spontaneously (Iriti and Faoro, 2003), those can be responsible for activating genes that determine the defense mechanism against the expansion of injury (Gravano et al., 2004).

Inside de leaf O_3 induces the formation of pectin cell wall protrusions as an apoplastic reaction of this oxidative stress in many temperate species, this reaction has been described as an important marker for O_3 symptom validation (Paoletti et al., 2009; Vollenweider et al., 2003; Reig-Armiñana et al., 2004) and this marker was observed in both, field and experimental samples.

Usually, O_3 injury induce a hypersensitive response (HR), that consists of palisade mesophyll cells collapse (Guderian et al., 1985) as a result of the accumulation ROS. However, the palisade parenchyma of *A. graveolens* has a particular characteristic that

indicate its function as a protective barrier against high radiation. We believe this tissue has little metabolic function because the ultrastructure analysis identified chloroplasts of reduced size and few organelles as mitochondria and Golgi complex compared to those found in the spongy parenchyma. Based on that, we believe the reactions caused by the oxidative O_3 effects are different when *A. graveolens* is exposed to different environmental conditions.

Cell wall exudates may be considered a detoxification mechanism (Günthardt-Goerg et al., 1997) and on *A. graveolens* fumigated plants, the spongy parenchyma cells seem to play an important role on this detoxification process once the most prominent symptom occurs on this tissue as the production of a massive wart-like pectin protrusions and thickening cell walls.

The spongy parenchyma cells of asymptomatic field samples were not as healthy as the cells of the same tissue on leaves used as control in the experiment. The spongy parenchyma cells of field samples presented markers of degenerative process as increased number of plastoglobules and nucleus degeneration, probably due to the leaves ageing process. In this case, spongy parenchyma cells were not capable anymore to act efficiently in the detoxification process thus, ROS could interact directly with plasma membrane bound receptors triggering downstream events in the cytosol (Baier et al., 2005) and the HR-like effects on the palisade parenchyma cells occurred. The gradient of oxidize condensed tannins on palisade cells were particularly remarkable markers of O_3 stress (Günthardt-Goerg and Vollenweider, 2007) and this marker was very important for validation analyses in the field samples.

Clear evidences suggest that O_3 stress interact with light stress and the exposure to sun-light influences on the visible and microscopical O_3 symptom expressions (Vollenweider et al., 2003). Because of this, we believe that the intense solar radiation in the field contributed to the development of HR-like once, high radiation intensify ROS production in the chloroplasts contributing to the O_3 threshold to be reached and the process of programmed

cell death triggered. On fumigated samples the O_3 threshold was not reached and no HR-like occurred, although, is important to take into account that in a preliminary O_3 exposure to high level of O_3 (200 ppb h / 8h, data not show) HR-like occurred on palisade parenchyma cells, even with low radiation, showing that a O_3 threshold needs to be reached for HR-like induction.

The rounded appearance of chloroplasts, with many plastoglobules, observed in *A. graveolens* symptomatic samples has been described in species of temperate region (Pääkkönen et al., 1995), as markers of oxidative stress resulting from O_3 but also of senescence processes. According Mikkelsen-Heide and Jorgensen (1996), the increase in plastoglobules reflects translocation of materials stemmed from the thylakoids, and the increase in size is probably due to the melting of small plastoglobules.

It is important to take into account that structural changes in chloroplasts may be reflecting the effects resulting from modifications such as, changes in the permeability of membranes, osmotic conditions and failures in the energy supply (Holopainen et al., 1992). However, Bréhélin et al. (2007), raise the possibility that changes in plastoglobules are related to the increased production of small molecule antioxidants such as tocopherols, since plastoglobules are directly related to the synthesis and storage of these molecules. According to these authors, the tocopherols are capable of protecting lipid membranes and photosystem II photoinactivation when transported to the thylakoid membranes, which neutralize the ROS produced, therefore, the relation between decreasing values of P_n e g_{wv} and the induction of visible symptoms during fumigation, suggest drastic effects on chloroplast function as a reaction to the oxidative stress.

P. gonoachanta

P. gonoachanta presented a high sensitivity to O_3 when exposed to controlled conditions, with a fast shedding process, with typical choloris occurring and also the formation of specific O_3 symptoms visual symptoms. However in the field, we believe the quantification of the visual symptoms was underestimated considering most of the symptomatic leaves felt along the season.

Leaf choloris was remarkable during O_3 fumigation and abundantly found in the field, but in fact, the chorosis occurs due to an accelerated cell senescence process (ACS), defined as a slowly process that occurs in most leaf tissue, identical to the ageing process but that occurs in the youngest tissue and cannot be considered as a specific O_3 symptom (Vollenweider et al., 2003; Günthardt-Goerg e Vollenweider, 2007). Thus, only the stippling, which occurs due to the HR-like process were considered in the field quantification. Other types of visual symptoms observed on *P. gonoachanta* can be confused as O_3-like (annex 2) and the validation must be made based on microscopical observation.

According Vollenweider et al. (2003), a HR-like is a localized defense reaction characterized by the induction of specific structures in discrete groups of quickly dying cells. In *P. gonochanta* this reaction was mainly responsible for the appearance of visible damage, and the structural markers found confirm the nature of the reaction, demonstrating the great sensitivity of the species to the effects of O_3.

The palisade parenchyma of this species is the principal, photoactive tissue, presenting large chloroplasts, many mitochondria and a large homogeneous nucleolus when compared to spongy parenchyma cells. According to Mittler et al. (2004), the membranous organelles featuring intense metabolic activity and high rate of flow of electrons, are the principal arrays of ROS production in plant cells, so they are the first to suffer ROS effects.

The reduction in photosynthetic capacity, due to the chloroplast disrupting, can contribute to the induction of senescence due to ROS formation (Eckardt and Pell, 1994). We believe the intense oxidative effect in *P. gonoachanta* were due to the accumulation of high levels of ROS inside the leaves that trigged the formation of visual symptom and also accelerate the shedding process.

C. floribundus

C. floribundus did not present specific visual symptoms on experimental conditions thus, this species cannot be used as a bioindicator of O_3 once only presented an accelerated shedding. Indeed, O_3 can accelerate the loss of older leaves and stimulate the production of new foliage (Pell et al., 1994).

O_3 exposures induce decreases in gas exchange rates during the oxidative process (Gravano et al., 2004; Novak et al., 2005), but, fumigated *C. floribundus* presented no significant differences in P_n e g_{wv} values when compared to control plants, which indicates the high tolerance of this species to the effects of O_3. The gas exchange rates decreased only when the leaves was into senescence process.

Bortier et al. (2000), suggested that faster growing species trend to be more sensitive to O_3 than slower growing species, but *C. floribundus* is a fast-growing pioneer species (Guaratini et al., 2008) which has seemed to have an antioxidant metabolic potential to be used in the detoxification process.

The O_3 tolerance can be related to two main points: the O_3 uptake and the antioxidant potential of each species. Once we know the gas exchange was uninterrupted in *C. floribundus*, we believe that this species has a great antioxidant capacity, once it is a pioneer species very well adapted to high radiation levels. Besides, the species present a dense trichomes barrier on the abaxial surface which may protect the leaves against the O_3 entrance.

Conclusion

The tropical species tested are sensible to O_3 oxidative stress, although only *A. graveolens* and *P. gonoachanta* presented specific visual symptoms able to be validated in the field sample by means of structural marks. The high biodiversity found in the rain forest may contribute to elucidate how responses caused by O_3 may triggered different defense pathways which were very specific for each species tested.

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Annex I

Visual symptoms not related to oxidative stress in leaves of *A. graveolens.*

Annex II

Visual symptoms not related to oxidative stress in leaves of *P. gonoachanta*.

Capítulo 3

Acúmulo de H_2O_2 e morte celular programada (MCP) em três espécies nativas em decorrência da exposição ao O_3

Acúmulo de H_2O_2 e morte celular programada (MCP) em três espécies nativas em decorrência da exposição ao O_3

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Resumo

A região metropolitana de Campinas (RMC), SP apresenta níveis de ozônio troposférico (O_3) potencialmente tóxicos para a vegetação local. O O_3 pode intensificar a produção de peróxido de hidrogênio (H_2O_2) que, quando acumulado nos tecidos foliares, pode ativar o processo de morte celular programada (MCP), causando sintomas visíveis. Objetivou-se determinar por meio de testes histoquímicos a sensibilidade aos efeitos do O_3 em três espécies arbóreas nativas e ocorrentes em fragmentos florestais da RMC: *Astronium graveolens* - Anacardiaceae, *Piptadenia gonoacantha* - Fabaceae e *Croton floribundus* - Euphorbiaceae. Mudas das três espécies foram fumigadas com O_3 e amostras com e sem sintomas foram analisadas quanto ao acúmulo de H_2O_2 (3,3'-diaminobenzidina-DAB) e MCP (Azul de Evans). Amostras com sintomas similares aos da fumigação foram coletadas em fragmentos florestais na RMC e também analisadas quanto ao acúmulo de H_2O_2 e MCP. Em *A. graveolens* fumigado ou coletado em campo houve acúmulo de H_2O_2, mas MCP ocorreu apenas nas amostras do campo. Folhas de *P. gonoachanta* fumigadas e do campo apresentaram acúmulo de H_2O_2 e MCP. *C. floribundus* não apresentou marcação histoquímica específica. As três espécies apresentam diferentes tipos de reação quando expostas ao O_3: *A. graveolens* e *P. gonoachanta* são espécies sensíveis e apresentaram respostas semelhantes na fumigação e no campo, enquanto *C. floribundus* não apresentou marcadores específicos quando exposto ao O_3.

Introdução

O ozônio (O_3) é um poluente altamente tóxico que causa mais danos à vegetação natural e culturas agrícolas do que qualquer outro poluente (Paoletti et al., 2010b; Matyssek et al., 2012).

A capacidade das plantas de reagir metabólica, fisiológica, morfológica e estruturalmente a mudanças nas concentrações de poluentes atmosféricos, especialmente o O_3, possibilita determinar sua sensibilidade ou tolerância aos efeitos oxidativos provocados por tal poluente.

A concentração de O_3 no interior da folha, quando a mesma é exposta a esse gás, é próxima de zero (Laisk et al., 1989), o que significa que o poluente é rapidamente degradado sendo produzidas espécies reativas de oxigênio (ERO) como o oxigênio singleto ($^1O_2$), o anion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (HO●), que são altamente reativas e tóxicas e podem causar a oxidação de constituintes celulares (Mittler et al., 2004).

Uma rede intrincada de defesa e reparo neutraliza estas reações de oxidação. As mais importantes enzimas que neutralizam as ERO em plantas incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT), glutationa peroxidase (GPX) e peroxiredoxina (PrxR). Juntamente com os antioxidantes não enzimáticos como o ácido ascórbico (AA) e a glutationa reduzida (GSH). Estas enzimas fornecem às células um maquinário altamente eficiente para a desintoxicação das ERO (Mittler et al., 2004).

O desequilíbrio entre a geração de ERO e a desintoxicação realizada pelos antioxidantes representa o estado metabólico referido como estresse oxidativo (Baier et al., 2005).

O H_2O_2 é uma ERO relativamente estável, sem carga elétrica, com livre difusão entre células (Iriti e Faoro, 2008) e que pode atuar no processo de sinalização, sendo capaz de

eliciar defesas antioxidantes ou, ainda, ativar o processo de morte celular programada (MCP), quando acumulado nos tecidos (Pellinen et al., 1999).

O processo de MCP induzido pelo O_3 é conhecido com resposta semelhante à de hipersensibilidade (HR-like), uma vez que os mecanismos envolvidos são semelhantes àqueles induzidos pelo ataque de patógenos (Pellinen et al., 1999). A desestruturação dos cloroplastos, juntamente com outros marcadores ultraestururais, permitem avaliar as modificações envolvidas nos processos de oxidação, para que a HR-like seja reconhecida como sendo causada por reações advindas da exposição ao O_3.

O acúmulo de H_2O_2 nos tecidos, bem como a MCP podem ser detectados por meio de testes histoquímicos, o que vêm contribuindo no entendimento do efeito do estresse oxidativo em tecidos vegetais expostos ao O_3. Além disso, tais testes permitem identificar precocemente esse efeito em folhas macroscopicamente assintomáticas (Faoro e Iriti, 2005; Iriti et al., 2003), contribuindo para o estabelecimento da sensibilidade de espécies ao O_3.

O presente estudo objetivou estabelecer, por meio de testes histoquímicos e análises ultraestruturais, a sensibilidade ao O_3 de três espécies nativas, frequentemente encontradas em fragmentos florestais presentes na Região Metropolitana de Campinas-SP, contribuindo para o conhecimento dos efeitos do O_3 em plantas nativas.

Material e métodos

Testes histoquímicos - Experimento de fumigação e coleta em campo

Os testes histoquímicos foram realizados em: *Astronium graveolens* Jacq. (Anacardiaceae), *Croton floribundus* Spreng. (Euphorbiaceae) e *Piptadenia gonoacantha* (Mart.) Macbr. (Fabaceae), e as metodologias utilizadas no experimento de fumigação e nas coletas em campo são descritas em Moura (2013, capítulo 2).

A análise para visualização do acúmulo de H_2O_2 foi realizada em, pelo menos, cinco amostras de três diferentes folhas de três plantas de cada espécie, submetidas à fumigação com O_3 e também em amostras sintomáticas e assintomáticas coletadas em campo.

Para a realização desse teste, amostras de folhas frescas com cerca de $1cm^2$ foram imersas em solução contendo 1mg mL^{-1} de 3.3'-diaminobenzidina (DAB)-HCl, (pH 5,6 ajustado com hidróxido de sódio a 1%); estas foram incubadas em câmara escura por vinte e quatro horas, em seguida, foram clarificadas em álcool a 96% (Iriti et al., 2003). As células com acúmulo de H_2O_2 apresentaram coloração marrom. Como controle negativo acrescentou-se 10 mM de ácido ascórbico à solução de DAB.

A detecção da MCP também foi realizada nos indivíduos submetidos à fumigação e nos coletados no campo. Para tanto, amostras de folhas frescas com cerca de $1cm^2$, com e sem sintomas visíveis, foram coletadas nas mesmas folhas selecionados para a análise de acúmulo de H_2O_2. As amostras foram aquecidas por um minuto em mistura de ácido lático, fenol, glicerina e água (1:1:1:1), contendo 20 mg mL^{-1} de azul de Evans, e em seguida clarificadas em álcool a 95% (Iriti et al., 2003, modificado- clarificação em álcool a 95%). As células mortas foram evidenciadas pela coloração azul, contrastando com as células sadias que se apresentaram transparentes.

Análises em microscopia eletrônica

As análises ultraestruturais em microscopia eletrônica de transmissão foram realizadas em *A. graveolens* e *P. gonocachanta.* A metodologia utilizada no preparo das amostras foi descrita por Moura (2013, capitulo 2), tendo sido observadas amostras sintomáticas e assintomáticas de ambas as espécies.

Análises da superfície foliar em microscopia eletrônica de varredura foram realizadas nas três espécies, embora sejam mostrados apenas resultados de *C. floribundus*, uma vez que

as outras duas espécies não mostraram resultados dignos de nota. Para as análises, amostras de folhas sintomáticas e assintomáticas oriundas do experimento de fumigação foram secas ao ponto crítico com acetona em equipamento Bal-Tec CPD-030, aderidas em suportes metálicos com fita dupla face, metalizadas em ouro em metalizador Bal-Tec SCD 050 e observadas em microscópio de varredura Philips XL series XL 20.

Resultados

Testes histoquímicos

Em *A. graveolens* o corante Azul de Evans evidenciou MCP apenas em amostras coletadas no campo, que apresentaram células do parênquima paliçádico coradas de azul (Fig. 1A-B). O acúmulo de H_2O_2 foi observado tanto nas amostras fumigadas (Fig. 1C) como nas amostras coletadas no campo (Fig. 1D), ocorrendo em células do parênquima paliçádico que apresentaram coloração marrom intensa.

Em *P. gonoachanta* observamos MCP, presente em células do parênquima paliçádico (Fig. 2A-B), nas células guarda dos estômatos (Fig. 2C-D), tanto em amostras fumigadas, quanto nas coletadas em campo. Nas amostras fumigadas foi possível verificar acentuada MCP nas células do pulvino (Fig. 2H). O acúmulo de H_2O_2 nas células do mesofilo também foi observado em amostras fumigadas (Fig. 2E) e coletadas em campo (Fig. 2G). Nas amostras fumigadas o acúmulo de H_2O_2 também ocorreu na parede das células epidérmicas (Fig. 2G) e no pulvino (Fig. 2I).

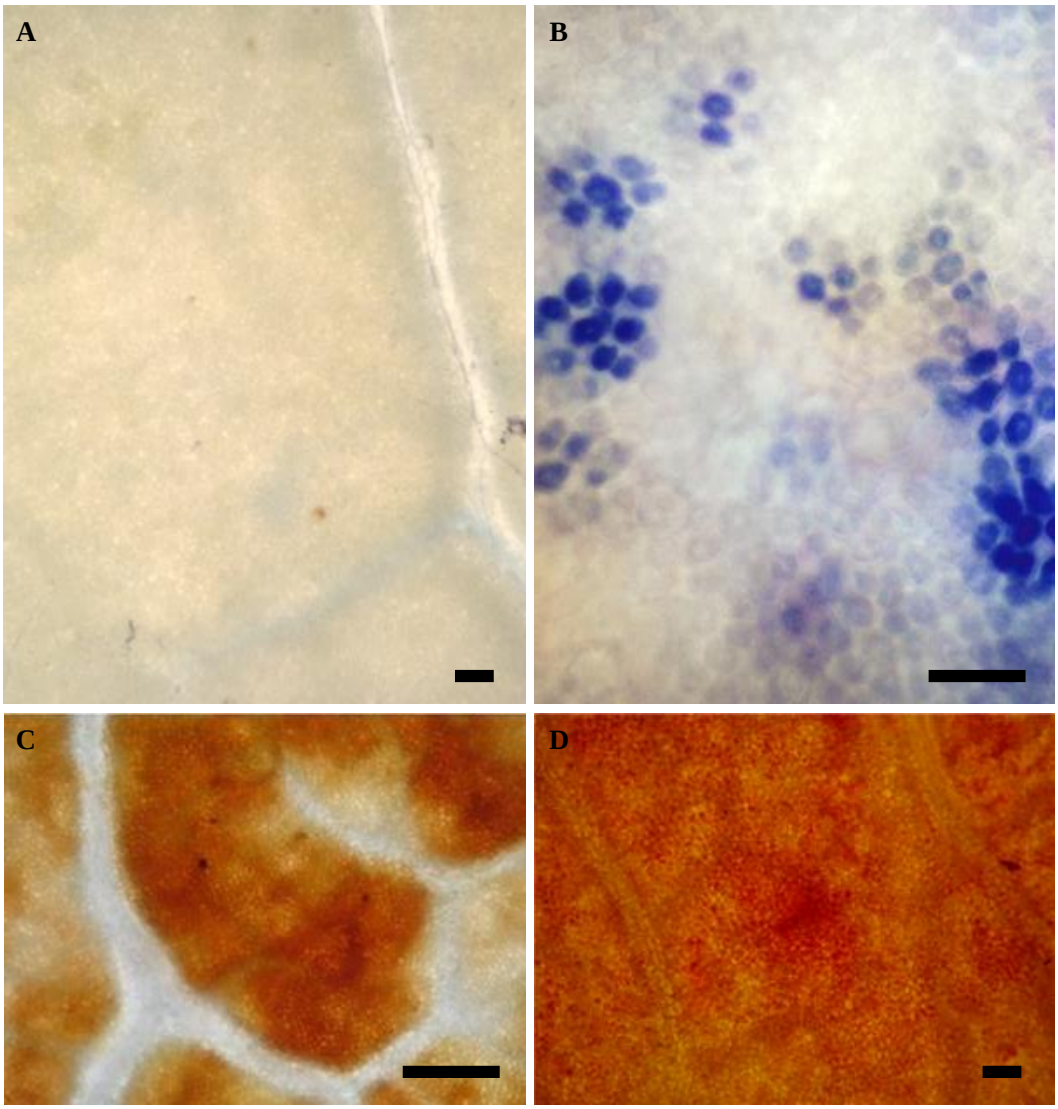

Figura 1. Testes histoquímicos em amostras foliares de *A. graveolens*. A. Resultado negativo para MCP em amostra fumigada. B. Resultado positivo (células coradas em azul) para MCP em amostras do campo. C. Acúmulo de H_2O_2 em amostras fumigadas. D. Acúmulo de H_2O_2 em amostras coletadas no campo. Barra = 150 µm.

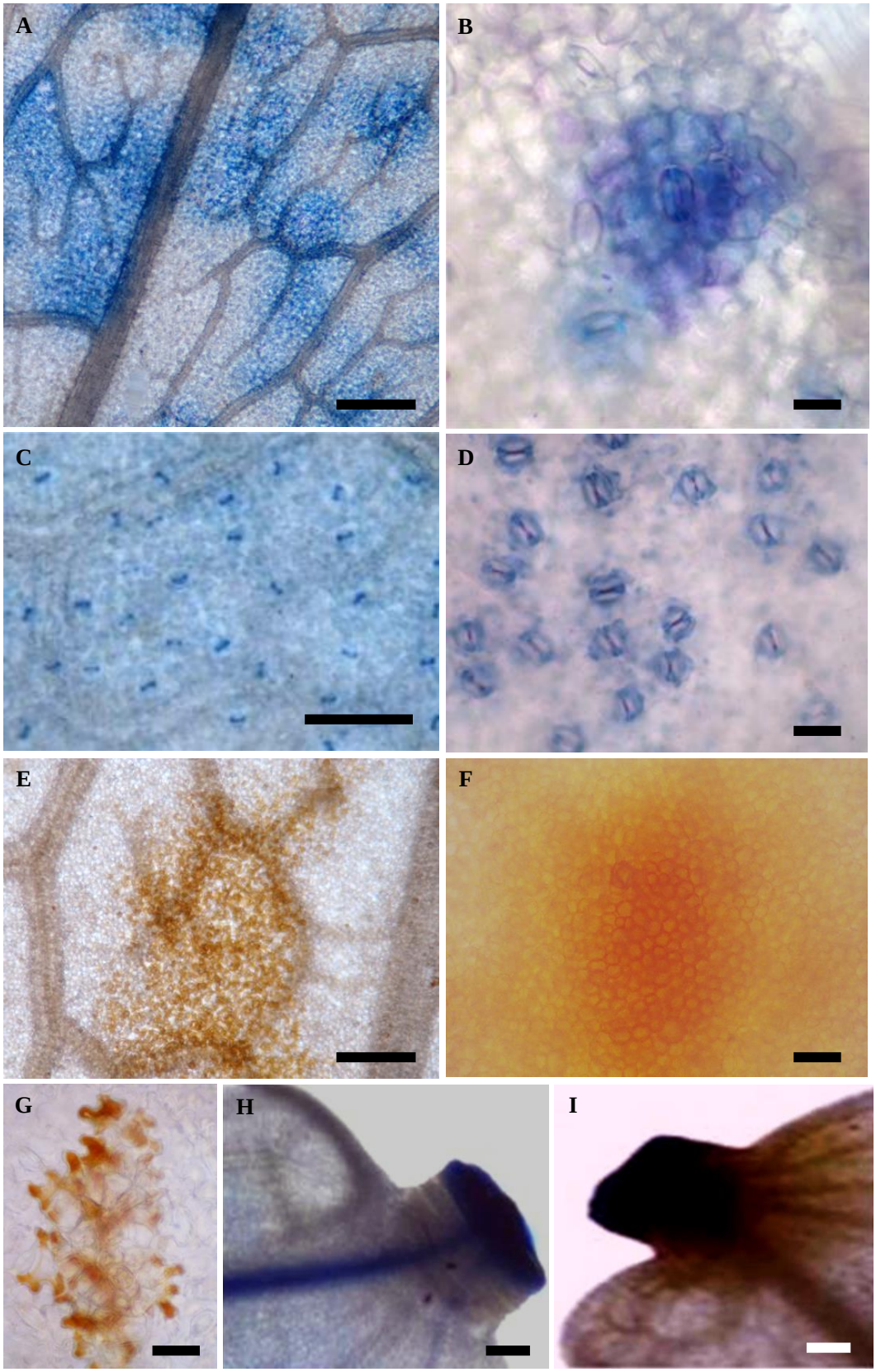

Figura 2. Testes histoquímicos em amostras foliares de *P. gonoachanta*. A e B. MCP marcada nas células do parênquima paliçádico em amostra fumigada. (A) e coletada no campo (B). C e D. MCP nas células guarda dos estômatos em amostra fumigada (C) e coletada no campo (D). E e F. Acúmulo de H_2O_2 nas células do parênquima paliçádico em amostra fumigada (E) e coletada no campo (F). G Acúmulo do H_2O_2 na parede das células epidérmicas em amostra fumigada. H. Acúmulo de H_2O_2 em células do pulvino em amostra fumigada. I.

MCP marcada nas células do pulvino em amostra fumigada. A, C, E, H, I, barra = 150 µm; B, D, F, G, barra = 30 µm.

Em *C. floribundus*, durante o experimento de fumigação e nas coletas em campo, não observamos sintomas específicos. Os resultados do teste com o Azul de Evans foram duvidosos, uma vez que a espécie é recoberta por uma densa camada de tricomas (Fig. 3A-B) inseridos no mesofilo de tal forma que permitiram a infiltração das soluções mesmo em amostras controle (Fig. 3C). Não houve acúmulo de H_2O_2 em nenhuma das amostras analisadas (Fig. 3D)

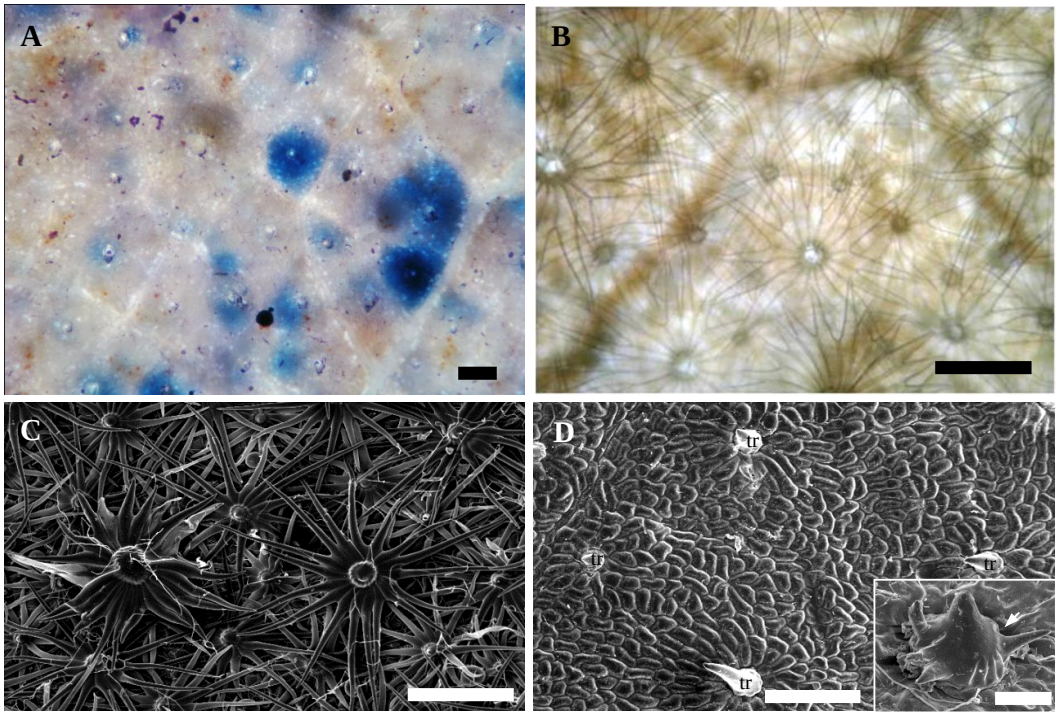

Figura 3. Testes histoquímicos e microscopia eletrônica de varredura em amostras foliares de *C. floribundus*. A. A marcação de MCP ocorre sempre próxima aos tricomas em amostra controle do experimento. B. Acúmulo de H_2O_2 não é evidenciado em amostras fumigadas. C. Densa camada de tricomas que recobre a superfície abaxial. D. Tricomas que atravessam o mesofilo (tr) formam uma lacuna em seu ponto de inserção (detalhe) onde acreditamos que o corante Azul de Evans fica retido. barra = 150 µm (detalhe em D barra = 40 µm).

Análises ultraestruturais

No apoplasto de amostras sintomáticas de *A. graveolens* e *P. gonocachanta*, observamos a formação de protrusões pécticas nas paredes celulares (Fig. 4), sendo que tal reação foi muito mais intensa em *A. graveolens*, principalmente nas amostras sintomáticas provenientes do experimento de fumigação.

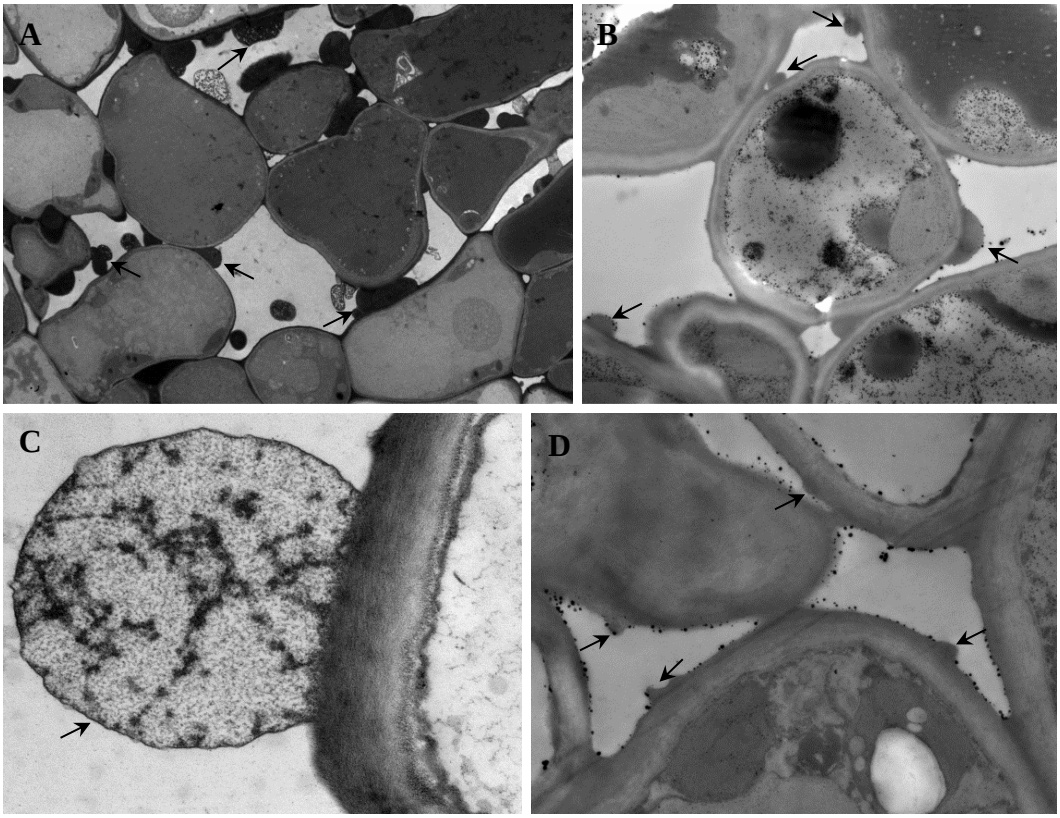

Figura 4. Protrusões pécticas decorrentes do estresse provocado pelo O_3 (seta). A-B. *A. graveolens*. A. amostra fumigada. B. amostra coletada em campo. C-D. *P. gonoachanta*. C. amostra fumigada. D. amostra coletada em campo. Magnitude das imagens: C = 33000X; D = 13500; B = 9700X; A = 3400X.

No simplasto, em ambas as espécies coletadas no campo e também em *P. gonoacantha* fumigada, observamos a resposta semelhante a de hipersensibilidade (HR-like). Tal reação foi caracterizada por um rápido colapso celular com a morte das células envolvidas, no entanto, as organelas permaneceram parcialmente distinguíveis no interior destas células, sendo

possível visualizar facilmente a desestruturação dos cloroplastos (Fig. 5A-C) e a condensação da cromatina (Figs. 5A, 6A).

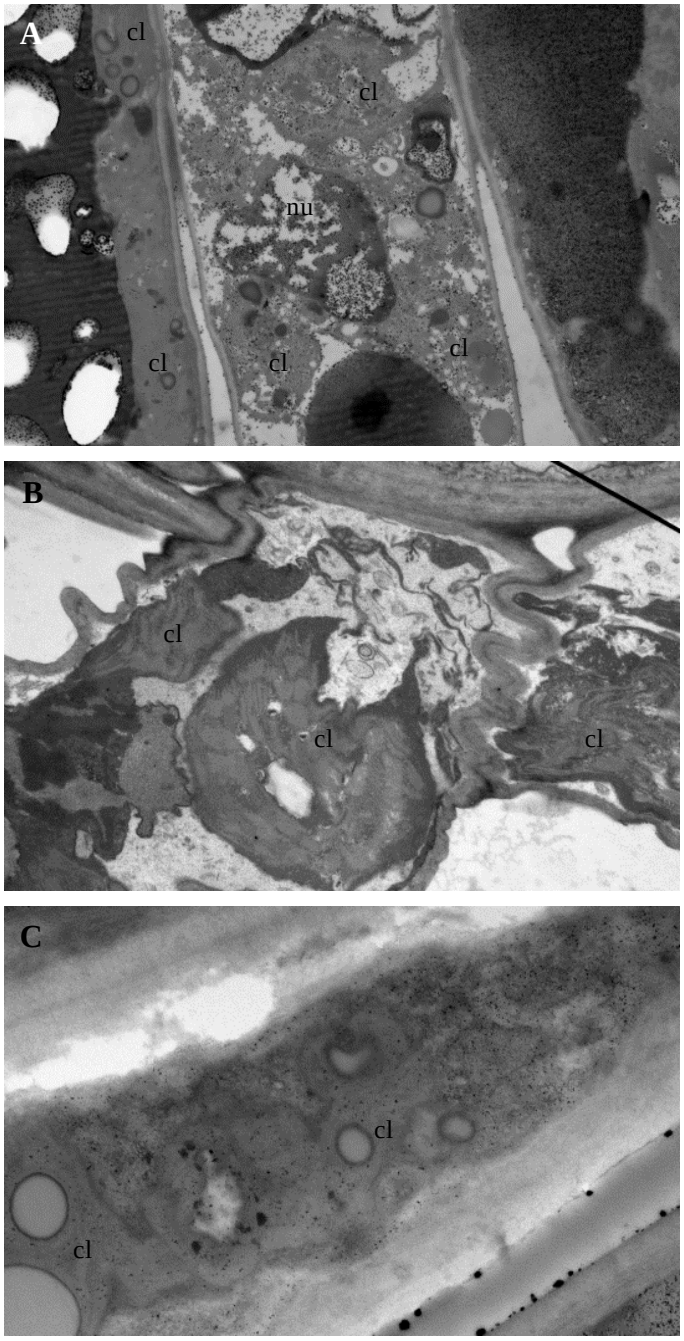

Figura 5. Reação do tipo HR-like. A. amostra sintomática de *A. graveolens* coletada em campo, note a condensação da cromatina no núcleo (nu) e os cloroplastos (cl) desestruturados na célula em HR-like localizada no centro da imagem. B. Amostra sintomática de *P. gonoachanta* fumigada com células em processo de HR-like que ocorrem no local da pontuação, note a desestruturação dos cloroplastos. C. Amostra sintomática de *P. gonoachanta* coletada em campo com células em processo avançado de HR-like onde os cloroplastos são praticamente indistinguíveis. C = 24500X; B = 13500; A = 97000X.

Em amostras sintomáticas de *P. gonoachanta* ocorreu clorose intensa e a analise ultraestrutural das células cloróticas revelou um aumento significativo no número e tamanho dos plastoglóbulos, que estavam sendo exportados para o vacúolo (Fig 6 C-F). Além disso, o núcleo se mostrou bem estruturado, com aspecto homogêneo e aparentemente funcional quando comparado com o das células em processo de HR-like (Fig. 6A-B), assim como os cloroplastos (Fig. B) e as mitocôndrias (Fig. 6A) que também se mostraram aparentemente funcionais.

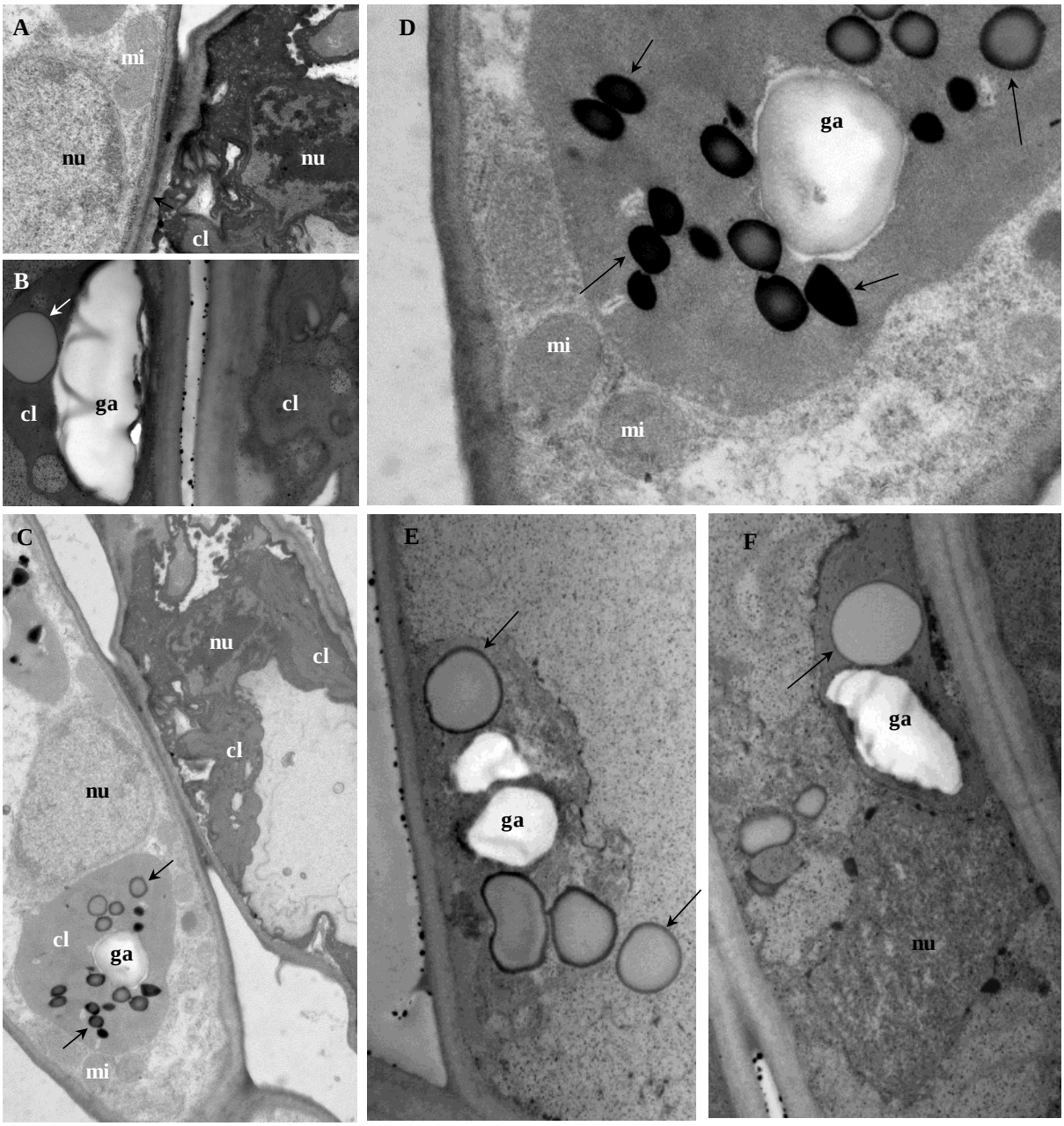

Figura 6. Diferenças entre células em processo de HR-like e em senescência em amostras sintomáticas de *P. gonoachanta*. A, C e D. Amostras fumigadas. B, E e F. Amostras do campo. Nas células em processo de HR-like os cloroplastos (cl) estão desestruturados (A, B e C), não há acúmulo de plastoglóbulos e o núcleo (nu) apresenta condensação da cromatina. Nas células em processo de senescência (A, B e C, células à esquerda das imagens e D, E e F) os cloroplastos continuam com grana distinguível (D), são encontrados muitos plastoglóbulos (setas) sendo exportados para o vacúolo (E), existem de grãos de amido (ga), o núcleo não esta condensado (F) e muitas mitocôndrias (mi) estão presentes (D). Magnitude das imagens: A, B, D = 24500X; E, F = 17500X; C = 9700X

Discussão

Dentre as ERO, o H_2O_2 é o oxidante mais estável e sem carga elétrica (Biernet et al., 2007), o que lhe confere capacidade de se difundir rapidamente através da membrana celular. Dessa forma, o H_2O_2 tem sido considerado uma molécula sinalizadora (Apel e Hirt, 2004) que pode eliciar as defesas antioxidativas (Dizengremel et al., 2008) ou ativar o processo de MCP (Rao e Davis, 2001) dependendo da sensibilidade da espécie.

O H_2O_2 gerado na membrana plasmática, ou extracelularmente no apoplasto, pode ser produzido a partir de peroxidases da parede celular dependentes do pH, que são ativadas em pH alcalino, que, na presença de um redutor, produzem H_2O_2 (Gill e Tuteja 2010). A alcalinização do apoplasto, em consequência do reconhecimento de um eliciador, precede a explosão oxidativa (oxidative burst), sendo que tal processo tem sido proposto como um meio alternativo de produção de ERO durante o estresse biótico (Gill e Tuteja 2010).

As ERO oxidam constituintes celulares como os lipídeos, proteínas e ácidos nucleicos e podem iniciar reações de sinalização em cadeia. Quando os níveis de ERO não excedem a capacidade antioxidante do apoplasto, as células podem reagir apenas localmente e não há indução da MCP. Durante o experimento de fumigação, *A. graveolens* apresentou acúmulo de H_2O_2 que acreditamos não ter excedido a capacidade antioxidante do apoplasto, uma vez que, em situação controlada, não foi observada a MCP e sim uma intensa reação oxidativa no apoplasto, que levou à formação dos sintomas visíveis caracterizados como “stipplings” (Moura, 2013, capitulo 2).

Quando a capacidade de defesa no apoplasto é ultrapassada há a indução de respostas endógenas que ativam a geração de ERO no simplasto, induzindo a MCP (Kangasjärvi et al., 2005). Acreditamos que essa capacidade de defesa no apoplasto foi excedida em *A. graveolens* coletado em campo, acarretando efeitos oxidativos mais intensos. Tais efeitos podem estar relacionados com a alta radiação solar registrada no campo (Moura, 2013, capítulo 1), que intensifica o efeito oxidativo provocado pelo O_3 (Paoletti et al., 2010a), uma vez que organelas com atividade metabólica altamente oxidante ou com taxa de fluxo de elétrons intensa, tais como cloroplastos, mitocôndrias e peroxissomos, são as principais fontes de produção de ERO nas células vegetais (Mittler et al., 2004).

Iriti et al. (2006), ressaltam que a MCP esta sempre associada à presença de depósitos de H_2O_2, assim como observado em *P. gonoacantha*, e esse acúmulo parece estar ligado diretamente a indução da MCP. As pontuações que caracterizam os sintomas visíveis encontrados, tanto durante a fumigação quanto na coleta em campo, são decorrentes de HR-like (Moura, 2013, capítulo 2), tendo sido marcadas, em ambos os casos, pelo azul de Evans. Segundo Iriti (comunicação pessoal) o corante Azul de Evans é capaz de penetrar apenas em células que apresentam degradação nas membranas, sendo assim um bom indicador de MCP.

Em experimento in vitro, onde a abscisão foliar foi induzida pelo estresse salino em folhas de *Capsicum chinense* (Sakamoto et al., 2008), uma contínua produção de H_2O_2 em células foliares localizadas na zona de abscisão foi revelada por marcadores histoquímicos em análises microscópicas, demonstrando que em situação de estresse o acúmulo de H_2O_2 esta envolvido diretamente na degradação das paredes celulares favorecendo a abscisão foliar. O acúmulo de H_2O_2 em *P. gonoacantha* também parece ter agido como um sinalizador que eliciou a MCP das células do pulvino, o que induziu a intensa e rápida queda dos folíolos (Moura, 2013, capítulo 2).

O fenômeno de indução da MCP reúne muitas características fisiológicas e moleculares que foram visualizadas por meio das análises ultraestruturais das amostras das duas espécies.

É comum ocorrer um mosaico de sintomas no tecido exposto ao O_3, com células em processo de HR-like, o que ocorreu nas áreas das folhas com pontuações; e com células em processo de senescência celular acelerada (SCA), que ocorreu nas áreas cloróticas. É importante levar em consideração que a sinalização proveniente das células que passaram pelo processo de necrose, causada pelas ERO diretamente formadas pelo contato com o O_3, pode desencadear o processo de MCP nas células vizinhas. (Kangasjärvi et al., 2005). Acreditamos que o mosaico de sintomas que ocorreu em *P. gonoachanta* demonstra claramente como a resposta aos efeitos do O_3 é dinâmica e os processos de sinalização desencadeados pelas ERO são essenciais para que uma determinada resposta específica ocorra.

Embora testes bioquímicos não tenham sido realizados no presente estudo, em *C. floribundus* a ausência de sintomas visíveis específicos e de marcações histoquímicas especificamente ligadas ao processo de estresse por O_3, sugerem que esta espécie apresenta mecanismos de desintoxicação eficientes, que podem estar ligados à produção de antioxidantes, tanto enzimáticos como não enzimáticos. Além do possível alto potencial antioxidante da espécie, a densa camada de tricomas que recobre a superfície abaxial pode ser considerada uma barreira mecânica, na qual o O_3 é quebrado antes de entrar na folha.

Conclusão

Com as análises ultraestruturais foi possível visualizar com clareza a reação apoplastica em *A. graveolens* e *P. gonoachanta* e diferenciar com exatidão as células em processo de HR-like das células em processo de senescência, o que contribuiu para a determinação da sensibilidade das espécies testadas.

Considerando os testes realizados e as observações conduzidas, concluímos que *P. gonoachanta* é, dentre as espécies testadas, a mais sensível, uma vez que o acúmulo de H_2O_2 desencadeou o processo de MCP, tanto nas amostras utilizadas no experimento quanto

naquelas coletadas em campo. *A. graveolens* também é muito sensível ao estresse provocado pelo O_3, no entanto a indução da MCP devido ao acúmulo de H_2O_2 parece estar relacionada com a exposição das folhas a alta radiação luminosa, enquanto que sob radiação luminosa mais baixa, o processo de desintoxicação no apoplasto parece ser eficiente não ocorrendo HR-like. *C. floribundus* não apresenta nem acúmulo de H_2O_2 nem MCP, portanto acreditamos que esta é uma espécie menos sensível aos efeitos do O_3, possivelmente por apresentar um sistema antioxidante muito eficiente.

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Conclusões e considerações finais

Com o presente estudo monstramos que os níveis de O_3 registrados na Região Metropolitana de Campinas/SP, Brasil, são altos o suficiente para causar danos oxidativos na vegetação. Tais níveis podem ser ainda mais prejudiciais à vegetação de clima tropical, quando comparados com a vegetação de clima temperado, uma vez que na região estudada valores elevados de O_3 foram registrados o ano todo. Nas regiões tropicais as condições favoráveis para o crescimento da vegetação, para a maioria das espécies, também estão presentes durante o ano todo, uma vez que, mesmo no inverno, as condições climáticas não são restritivas ao crescimento, diferentemente do que ocorre nas espécies folhosas de regiões temperadas, que perdem as folhas no inverno.

Sintomas visíveis específicos do estresse provocado pelo O_3 foram validados com base em marcadores estruturais e ultraestruturais em duas das três espécies testadas, *A. graveolens* e *P. gonoachanta*, e ambas mostraram diferenças quanto ao tipo de resposta apresentada. Diante disso, e considerando a alta biodiversidade encontrada na região, certamente existem muitas estratégias a serem elucidadas sobre como o O_3 desencadeia os processos de defesa em espécies nativas tropicais.

Dentre as espécies testadas, *P. gonoachanta* apresentou maior sensibilidade aos efeitos danosos provocados pelo O_3. *A. graveolens* também se mostrou muito sensível, no entanto a espécie aparente possuir grande capacidade de desintoxicação ainda no apoplasto e a indução da HR-like parece estar relacionada com a exposição das folhas a alta radiação luminosa, que potencializa o efeito oxidativo do O_3. Acreditamos que *C. floribundus* é uma espécie menos sensível aos efeitos do O_3, possivelmente por apresentar um sistema antioxidante muito eficiente.

O presente estudo abre um leque de possibilidades para futuros projetos. Estudos sobre o fluxo de O_3 em espécies tropicais permitirão estabelecer a dose necessária para provocar

sintomas visíveis nas espécies estudadas, contribuindo para o melhor entendimento dos efeitos oxidativos provocados pelo O_3 em espécies tropicais.

Estudos que permitam analisar a capacidade antioxidante das espécies também serão necessários. O estudo dos níveis de antioxidantes que atuam no apoplasto de *A. graveolens* ajudaria a entender a dinâmica dos efeitos do O_3 quando este ainda esta causando a degradação das paredes celulares, antes que a dose limite seja alcançada, dose essa que potencialmente é a causa dos danos no simplasto. O estudo do potencial antioxidante de *C. floribundus* também é necessário, uma vez que nossos resultados levam a crer que a espécie possui um sistema de desintoxicação muito eficiente, que lhe confere menor sensibilidade ao O_3.

A relação entre o acúmulo de H_2O_2, MCP e senescência dos folíolos de *P. gonoachanta* sugere que uma melhor avaliação das células da região do pulvino seja interessante para o entendimento do processo de queda acelerada de folhas devido ao efeito oxidativo provocado pelo O_3.

No campo, um monitoramento minucioso da formação dos sintomas visíveis em *P. gonoachanta* e *A. graveolens* se faz necessário para entendermos melhor o impacto do O_3 sobre a vegetação tropical e a interferência de outras variáveis climáticas na formação dos sintomas visíveis.

Acreditamos que no ambiente analisado ainda existam muitas espécies sensíveis ao O_3 e que apresentam sintomas característicos dos efeitos oxidativos provocados por esse poluente. A grande biodiversidade encontrada nos ecossistemas tropicais pode revelar muito sobre o potencial prejudicial do O_3 à vegetação e os mecanismos de defesa das plantas contra o mesmo.