FRANCINE FAIA FERNANDES

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Tese apresentada ao Instituto de Botânica da Secretaria de Infraestrutura e 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.

SÃO PAULO
2019

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Ficha Catalográfica elaborada pelo **NÚCLEO DE BIBLIOTECA E MEMÓRIA**

Fernandes, Francine Faia
F363c Características morfo-anatômicas foliares funcionais e respostas estruturais de diferentes grupos funcionais da Floresta Atlântica a distúrbios ambientais / Francine Faia Fernandes -- São Paulo, 2019.
110p.; il.

Tese (Doutorado) -- Instituto de Botânica da Secretaria de Infraestrutura e

Bibliografia.

1. Poluição atmosférica 2. Anatomia vegetal. 3. Estresse oxidativo.
I. Título.

CDU: 628.395

*Dedico à Dra. Edenise Segala Alves, e aos meus pais, José Carlos Flor Fernandes e Fátima Faia Fernandes*

“Na vida, não existe nada a temer, mas a entender.”

(Marie Curie)

**Agradecimentos**

À minha querida orientadora Dra. Marisa Domingos pela orientação dedicada, por confiar no meu conhecimento acadêmico-científico e por me oferecer meios e ideias para a elaboração desta tese. É com muita admiração e carinho que lhe agradeço por ter me aceito como aluna e, principalmente, por se preocupar com a minha formação.

À minha querida co-orientadora Dra. Poliana Cardoso-Gustavosn pela disponibilidade durante todo o período para tirar minhas duvidas, pelas contribuições valiosíssimas para a elaboração desta tese e por enxergar o valor do meu trabalho quando eu mesma não enxergava. Tenho muito orgulho de ter sido sua aluna e de ter seus ensimanetos como parte da minha formação.

Ao Programa de Pós-graduação em Biodiversidade Vegetal e Meio Ambiente, pelo ensino publico de qualidade.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de Doutorado.

À Fundação de Desenvolvimento da Pesquisa do Agronegócio (FUNDEPAG).

Ao Instituto de Botânica, em especial ao Núcleo de Pesquisa em Anatomia e ao Núcleo de Pesquisa em Ecologia por toda a infraestrutura e suporte concedidos.

Ao Institute for Sustainable Plant Protection/National Research Council of Italy por toda infraestrutura e suporte concedidos.

À minha supervisora Dra. Elena Paoletti por me receber em seu país, pelo seu interesse pela anatomia vegetal e oportunidade de trabalhar no sistema *Free-air $O_3$ exposure* ($FO_3X$).

À equipe que participou do experimento realizado no sistema $FO_3X$, em especial: Marcela Engela, Marisia Esposito, Bárbara Moura, Yasutomo Hoshika, Moreno Lazzara, Elisa Carrari, Alessandro Materassi, Giada Magni, Federico Brilli e Rita Baraldi.

A todos os integrantes do grupo que me acompanharam nas coletas e as fizeram mais divertida: Amariles, Giovanna, Marcela, Marisia, Ricks e Tiago. Vocês são incríveis e divertidos!

Ao Dr. Eduardo Pereira Cabral Gomes, Dr. Hildeberto Caldas de Sousa e Dra

Maria Cristina Teixeira Braga Messias e ao Dr. Marcos Enoque L. Lima pela colaboração na escolha das espécies arbóreas.

À Dra. Adriana H. Hayashi pela atenção, esclarecimentos técnicos e científicos durante a execução de análises laboratoriais e por viabilizar o desenvolvimento desse estudo no Núcleo de Pesquisa em Anatomia.

Ao funcionário e à ex-funcionária do Núcleo de Pesquisa em Anatomia, Newton e Maria.

À funcionária Marli e às ex-funcionárias Amariles e Dorinha, do Núcleo de Pesquisa em Ecologia.

Aos alunos e ex-alunos do Intituto de Botânica pelas festas, "*happy hour*" e momentos de descontração: Cássia, Camila, Diego, Douglas, Heloisa, Krysna, Laís, Marisia, Marina, Mayara, Milton, Omar, Regina, Richard, Ricardo, Rick Seven, Samantha, Simone, Sol, Thais, Vitinho, Victória de Carvalho, Vilmar e Zedenil. Em especial, Cleide, Leo, Lucas e Josi, pelas nossas reuniões de estudo proveitosas.

À Dra. Edenise Segala Alves, minha orietadora de mestrado que esteve presente em toda a minha vida acadêmica. Agreço pelo apoio durante todos esses anos e seus ensinamentos que foram de grande valia para o meu crescimento pessoal e profissional. Sou e sempre serei muito grata a você.

Aos meus grandes amigos pela parceria, conversas, risadas e viagens: Maria Cecília "Ciça", Gisele "Gisel", Giovanna "Gio", Débora "Debs", Vanessa "Van", Marcela "Marcthela", Josiane "Jo", Gustavo "Gu" e Igor.

E acima de tudo, à minha família, pelo apoio e incentivo em relação a minha carreria que escolhi trilhar. Em especial, os meus pais, pelo amor incondicional, pela educação e condições de estudo. Amo vocês!

**Resumo**

A Floresta Atlântica vem sendo alvo de perturbações causadas por poluentes atmosféricos e anormalidades climáticas. A interação entre diferentes estressores sob condições naturais pode promover efeitos sinérgicos nas plantas, respostas estas associadas à capacidade de dada espécie tolerar o estresse oxidativo. Entretanto, a definição de padrões de respostas ecossistêmicas nesse bioma brasileiro com base no potencial de aclimatação (tolerância) e suscetibilidade (sensibilidade) ao estresse oxidativo de suas espécies nativas é dificultada devido à sua alta biodiversidade. Assim, a presente tese utiliza como alternativa uma abordagem ecossistêmica, na qual as características morfo-anatômicas e as respostas estruturais (referidas como marcadores microscópicos) foliares de espécies representativas de três grupos funcionais da Floresta Atlântica (espécies arbóreas pioneiras, arbóreas não pioneiras e lianas) foram estudadas. Para tanto, duas etapas experimentais foram realizadas: (1) espécies arbóreas foram selecionadas e estudadas em remanescentes florestais localizados no sudeste do Brasil. Esses remanescentes florestais estão adaptados a diferentes condições climáticas e edáficas e também têm sido afetados por poluentes atmosféricos emitidos por diferentes fontes antrópicas; (2) *Passiflora edulis* (Passifloreaceae), espécie de liana, foi submetida ao ozônio em sistema FACE (*Free-Air Controlled Exposure*). Notou-se que, espécies arbóreas pioneiras possuem mesofilo mais compacto e maior densidade de tricomas, o que restringe ou evita os efeitos do estresse oxidativo causados por fatores de estresse naturais (por exemplo, alta radiação solar e déficit de pressão de vapor) e antrópicos (por exemplo, poluentes gasosos e particulados e mudanças climáticas), e, portanto, são potencialmente mais tolerantes que as espécies arbóreas não pioneiras. Ainda, as espécies representativas de cada grupo funcional (pioneira *vs* espécies arbóreas não pioneiras) foram agrupadas com base nas caracteristicas morfo-anatômicas funcionais foliares, as quais indicaram os níveis de potencial tolerância das espécies ao estresse oxidative (resultados apresentados no capítulo 1). Posteriomente, o nível de tolerância ao estresse oxidativo das espécies (espécies tolerantes, intermediárias e sensíveis) foi validado com base nos marcadores microscópicos encontrados. Espécies com menor potencial de tolerância apresentaram maior variedade desses marcadores no mesofilo, tais como: protrusões de parede, celulas parenquimáticas plasmolisadas, alterações na membrana celular e fragmentação do vacúolo em pequenas vesículas. As espécies potencialmente tolerantes, por sua vez, mostraram marcadores microscópicos

indicadores de aumento de resistência ao estresse oxidativo, como hipertrofia de células do mesofilo e acúmulo de fenólicos glicosilados no apoplasto (resultados apresentados no capítulo 2). Por fim, *P. edulis* mostrou-se também tolerante ao estresse oxidativo, uma vez que, na presença do ozônio, respondeu com senescência acelerada para evitar a propagação de danos, respostas estruturais e aumento dos compostos de defesa constitutiva. Tais respostas permitiram a aclimatação das plantas ao estresse oxidativo (resultados apresentados no capítulo 3). Esta tese enfatiza a impotância da anatomia para encontrar padrões de respostas das plantas e permitiu novos *insights* quanto a plasticidade de respostas das plantas à pressão oxidativa ambiental.

**Palavras-chave:** anatomia foliar**,** estresse oxidativo*,* filtros ambientais, marcadores miscoscópicos, morfologia foliar

**Abstract**

The Atlantic Rain Forest has been affected by air pollutants and climatic abnormalities. The interaction among different stressors under natural conditions might promote synergistic effects or cross-resistance in plants, which determined the ability or inability of a given species to tolerate the oxidative stress. However, the stablishment of ecosystemic patterns in this Brazilian biome based on the potential acclimation (tolerance) and susceptibility (intolerance) to oxidative stress of its native plant species is hampered by its high biodiversity. Therefore, this thesis applies an ecosystemic approach, in which the morpho-anatomical leaf traits and structural responses (here referred as microscopic markers) of species belonging to three functional groups of the Atlantic Forest (pioneer and non-pioneer tree species and lianas) were studied. In this regard, two experimental steps were performed: (1) tree species were selected and studied in forest remnants located in southeast Brazil. These forest remnants are adapted to different climatic and edaphic conditions and have also been affected by atmospheric pollutants emitted by different anthropic sources; (2) *Passiflora edulis* (Passifloreaceae), a liana species, was submitted to ozone in a FACE (*Free-Air Controlled Exposure*) system.We concluded that pioneer tree species have compact mesophyll and high trichome density, which restrict or avoid the effects of oxidative stress posed by natural (e.g. high solar radiation and vapor pressure deficit) and anthropic (e.g. gaseous and particulate pollutants and climatic changes) stressors. Therefore, pioneer species are more tolerant than non-pioneer species. In addiction, the representative species of each funtional group (pioneer *vs* non-pioneer tree species) were clustered with basis on their morpho-anatomical leaf traits, emerging groups of species with distinct potenctial tolerance levels to oxidative stress (results included in Chapter 1). Further, the tolerance level to oxidative stress was validated in the mentioned grups of tree species (tolerant, intermidiate and sensitive species) by using microscopic markers. Species lower potential of tolerance to environmental stress showed greater variety of microscopic markers in mesophyll, such as wart-like protrusion, plasmolysis of cells, plasma membrane changes and fragmentation of the central vacuole in numerous small vesicles. The tolerant species showed microscopic markers that are indicators of increased resistance against oxidative stress, such as hypertrophy of mesophyll cells and accumulation of phenolic glycosides in the apoplast (results included in Chapter 2). Finally, *P. edulis* showed also to be tolerant to oxidative

stress because, in the presence of ozone, plants responded with accelerated senescence to avoid the propagation of damage, structural responses and increase of constitutive defense compounds. These responses show a higher ability of plants to acclimate to oxidative stress (results included in Chapter 3). This thesis highlights the importance of plant anatomy to describe patterns of plant responses and new insights about the plasticity of different plant species to environmental oxidative pressure.

**Keywords:** environmental filters, foliar anatomy**,** foliar morphology, microscopic markers, oxidative burst

**Sumário**

## 1. Introdução geral e justificativas

### 1.1. Respostas morfo-anatômicas foliares em plantas submetidas a multiplos fatores de estresse ambiental

Os ecossistemas florestais estão sujeitos a diversos tipos de estresse causados por oscilações e anormalidades climáticas, como extremos de temperatura, déficit hídrico e excesso de radiação solar. Além disso, devido à expansão das cidades, das atividades agrícolas e industriais e das áreas de mineração, os ecossistemas naturais vêm sendo fragmentados e afetados por diferentes poluentes nas últimas décadas (Domingos *et al.,* 2003, 2015; Nakazato *et al.,* 2018). A Mata Atlântica, particularmente no sudeste brasileiro, vem sendo alvo de perturbações causadas por poluentes atmosféricos, como os óxidos de nitrogênio ($NO_x$) e enxofre ($SO_x$), material particulado (MP) e ozônio ($O_3$) (Domingos *et al.,* 2003, 2015; Brandão *et al.,* 2017; Esposito *et al.,* 2018; Nakazato *et al.,* 2018).

A entrada dos poluentes atmosféricos na vegetação se dá pela deposição seca (material particulado e/ou gases) ou úmida (na presença da precipitação) (Nagajyoti *et al.,* 2010), alcançando as folhas das plantas e o solo. Nas lâminas foliares (primeiro contato com poluição atmosférica) os poluentes: (1) são dissociados em meio aquoso, ou no caso da deposição úmida, entram diretamente pelas fissuras das cutículas (via de maior resistência); (2) entram pelos estômatos durante as trocas gasosas (esta última, considerada a via de menor resistência) (Roschina & Roschina, 2003; Shahid *et al.,* 2017).

O estresse oxidativo (*oxidative burst*) causado por condições climáticas e/ou poluição atmosférica é consequência do aumento da produção de espécies reativas de oxigênio (*reactive oxygen species*, ROS), entre elas superóxido ($O_2^-$), peróxido de hidrogênio ($H_2O_2$), radical hidroxila ($OH^-$) e oxigênio singleto ($^1O_2$) nos tecidos

vegetais, que podem ativar mecanismos de respostas envolvendo características bioquímicas, fisiológicas, estruturais e morfológicas (Bussotti, 2008; Cheng *et al.,* 2007; Fiscus *et al.,* 2005; Fuhrer & Booker 2003; Morgan *et al.,* 2006; Oksanen *et al.,* 2013; Ueda *et al.,* 2013).

As características foliares funcionais[1] são indicadoras úteis para o entendimento das estratégias de aclimatação/adaptação de plantas (Violle *et al.,* 2007; Díaz *et al.,* 2013; Bussotti & Pollastrini 2015; Nock *et al.,* 2016) que crescem naturalmente em locais afetados por diferentes fatores de estresse, que podem favorecer o aumento do estresse oxidativo em função do excedente de formação de ROS. As diferenças nas características funcionais, seja de um determinado indivíduo, ou de diferentes genótipos de uma mesma espécie, podem variar em consequência dos filtros ambientais; neste caso, tais características (*traits*) também podem ser chamadas de características de resposta ao estresse (*stress response traits*, Bussotti & Pollastrinni 2015) ou características de resposta (*response traits*, Violle *et al.,* 2007; Díaz *et al.,* 2013; Nock *et al.,* 2016). A amplitude com que uma determinada característica varia no mesmo organismo, é chamada de plasticidade fenotípica. Uma alta plasticidade fenotípica permite às espécies sobreviverem a uma ampla variedade de condições ambientais, reduzindo o risco de sua extinção em decorrência das mudanças climáticas (Bussoti & Polastrini 2015).

Em especial, as características morfológicas (*morphological leaf tratis*) e anatômicas (*anatomical leaf traits*), que incluem quaisquer caracteres relacionados à morfologia externa e anatomia das lâminas foliares, podem variar entre espécies e entre grupos funcionais de plantas (Poorter *et al.,* 2009), ao longo de um gradiente

[1]*Functional leaf traits* – conjunto de características (bioquímicas, fisiológicas e morfo-anatômicas) de um organismo que define o papel do mesmo no ecossistema (papel ecológico) ou performance (Bussotti & Pollastrini 2015).

ambiental[2], que podem funcionar como estratégias de aclimatação/adaptação a oscilações ambientais (Bussotti *et al.,* 2005; Bussotti 2008; Lusk *et al.,* 2008; Bussotti & Pollastrini 2015; Xu *et al.,* 2015). As variações nas características morfológicas e anatômicas podem ser induzidas, também, por estressores antrópicos como a poluição atmosférica. Por exemplo, o aumento na concentração de massa seca (*dry matter concentration*, DMC) já foi observado em gradiente longitudinal de estresse hídrico e $O_3$ (Bussotti *et al.,* 2005); variações na biomassa por área foliar (*leaf mass área*, LMA) já foram correlacionadas a variações na intensidade luminosa (Lusk *et al.,* 2008; Poorter *et al.,* 2009), temperatura, estresse hídrico, concentrações moderadas de $CO_2$ (Poorter *et al.,* 2009) e $O_3$ (Bussotti *et al.,* 2005); a espessura foliar (*leaf thickness*, LT) com o grau de aclimatação à alta radiação (BjörKman 1981); a área foliar específica (*specific leaf área*, SLA; que é o inverso da biomassa por área e expressa o desenvolvimento da superfície foliar por unidade de biomassa) foi correlacionada a elevadas concentrações de $NO_x$ (Jochner *et al.* 2015), $O_3$ (Pooerter *et al*., 2009; Calatayud *et al.,* 2011) e ao tamanho do MP depositado sobre a superfície foliar (Chen *et al.* 2015), a LT ao $O_3$ (Bussotti *et al.,* 2005b); o conteúdo relativo da água (*relative water contente*, RWC- índice que caracteriza o estado da água da folha) foi correlacionado negativamente ao tamanho do MP sobre a superfície foliar (Chen *et al.,* 2015); a densidade estomática (que expressa o número de estômatos por unidade de área foliar) à aclimatação (tolerância) ou suscetibilidade (intolerância) ao $O_3$ (Pääkönen *et al.,* 1997; Ferdinand *et al*., 2000; Moura & Alves 2014); a densidade de tricomas ao $CO_2$ e a sucetibilidade ao $O_3$ (Paoletti *et al.,* 2007) e a maior deposição de MP na superfície foliar (Zampieri *et al.,* 2013).

As alterações anatômicas nas plantas, denominadas marcadores microscópicos

[2] Gradiente ambiental - uma mudança gradual em um determinado fator ambiental biótico ou abiótico, por meio de espaço ou tempo (Garnier *et al.,* 2016).

estruturais e ultraestruturais, podem auxiliar na interpretação do nível de susceptibilidade de uma espécie vegetal aos fatores de estresse impostos pelas oscilações climáticas e atividades antrópicas (Vollenweider *et al*., 2003; Günthardt-Goerg & Vollenweider 2007; Moura *et al.,* 2018). Estes marcadores permitem, também, a detecção do estresse oxidativo, e das alterações na composição química dos tecidos foliares decorrentes de fatores abióticos (Kivimäenpää *et al*., 2003; Oksanen *et al.,* 2003; Vollenweider *et al.,* 2003; Gravano *et al.,* 2004; Günthardt-Goerg & Vollenweider 2006). Dentre os marcadores mais comuns alterados por diferentes poluentes ($O_3$ e/ou metais pesados), destacam-se o espessamento maciço das paredes celulares como resposta relacionada à desintoxicação (Günthardt-Goerg *et al.,* 1997; Vollenweider *et al.,* 2006; Moura *et al.,* 2018), protrusões nas paredes celulares voltadas para o apoplasto (estas, descritas mais comumente em resposta ao $O_3$–Günthardt-Goerg *et al.,* 1997, 2000; Vollenweider *et al.,* 2003; Günthardt-Goerg & Vollenweider 2007; Paoletti *et al.,* 2009; Pedroso *et al.,* 2016), alterações no metabolismo secundário da planta, com acúmulo e oxidação de compostos fenólicos (Günthardt-Goerg *et al.,* 2000; Vollenweider *et al.*, 2006; Guerreiro *et al.,* 2013; Kivimäenpää *et al.,* 2014; Fernandes *et al.*, 2016), alterações na autofluorescência das clorofilas (Günthardt-Goerg *et al.,* 1997; Schraudner *et al.,* 1998; Günthardt-Goerg & Vollenweider 2006; Vollenweider *et al.,* 2003, 2013; Pedroso & Alves 2015), aumento das lipofuscina (subproduto da peroxidação lipídica, Fernandes *et al.*, 2019), condensação dos cloroplastos e núcleo (Vollenweider *et al.,* 2006, 2013), o acúmulo de $H_2O_2$ (Gerosa *et al.,* 2009; Esposito *et al.,* 2018) e $O_2^-$ nos tecidos foliares (Esposito *et al.,* 2018). Algumas espécies podem apresentar um conjunto de marcadores que qualificam a resposta de hipersensibilidade (*hypersensitive like response*, HR-like) e de senescência celular acelerada (*accelerated cell senescence*, ACS) (Günthardt-Goerg & Vollenweider 2006; Vollenweider *et al.,*

2003). Essas alterações microscópicas são induzidas pelo estresse oxidativo decorrente dos poluentes e validam sintomas visíveis foliares supostamente causados por poluentes oxidativos como o $O_3$ (Günthardt-Goerg & Vollenweider 2006).

A resposta estrutural e a intensidade dos sintomas foliares visíveis variam, contudo, em função da grande plasticidade de resposta das plantas a múltiplos fatores de estresse ambiental que ocorrem simultaneamente em campo (Paoletti *et al.,* 2009). A interação entre os diversos fatores de estresse ambiental, como as variações climáticas e a poluição atmosférica, podem promover dois tipos diferentes de efeitos nas plantas, a resistência cruzada ou o sinérgico (Bussotti 2008). A resistência cruzada ocorre quando as defesas da planta, ativadas em resposta a um estressor ambiental, a protegem contra um segundo estressor ambiental, reduzindo os danos causados pelo último (Bussotti 2008; Paoletti *et al.,* 2009). O efeito sinérgico é caracterizado pela soma dos efeitos de estressores ambientais que intensificam a formação de ROS, promovendo maiores danos oxidativos (Foyer *et al.,* 1994; Paoletti *et al.,* 2009; Yamasaki *et al.,* 1997).

### 1.2. Avaliação das respostas estruturais em grupos funcionais submetidos à multiplos fatores de estresse ambiental

Ao se estudar os efeitos de estressores ambientais nas florestas tropicais e subtropicais, deve-se considerar sua alta biodiversidade, que dificulta a definição de padrões de respostas de aclimatação ou suscetibilidade em nível de ecossistema. Uma alternativa para aumentar a relevância ecológica do estudo é avaliar respostas indicadoras de aclimatação ou suscetibilidade ao estresse oxidativo em grupos

funcionais (*funtional group*[3]). Ecólogos utilizam o conceito de grupos funcionais em diferentes contextos, inclusive em estudos que buscam detectar, avaliar e prever mudanças ambientais, visto que é possível propor modelos menos complexos para prever respostas em nível ecossistêmico (Duckworth *et al.,* 2000; Gurevitch *et al.,* 2009). Por exemplo, segundo Favaretto *et al.,* (2011), as espécies arbóreas nativas das florestas tropicais podem ser classificadas em dois grandes grupos funcionais, com base na exigência de luz e aclimatação/adaptação ao sombreamento: espécies pioneiras, aquelas intolerantes ao sombreamento, e espécies não pioneiras, as aclimatadas/adaptadas ao sombreamento. Estudos recentes desenvolvidos em fragmentos de Floresta Atlântica em São Paulo indicam ser esta abordagem viável. Moura *et al.,* (2018) avaliaram as respostas morfológicas e anatômicas foliares frente ao $O_3$ de duas espécies pioneiras (*Croton floribundus*, *Piptadenia gonoacantha*) e uma não pioneira (*Astronium graveolens*) em ambiente controlado e ocorrentes naturalmente em fragmentos de Floresta Atlântica Semidecidual, na região metropolitana de Campinas. *C. floribundus* mostrou ser pouco afetado por $O_3$. Amostragens paralelas às realizadas por Moura *et al.,* (2018) em fragmentos de Floresta Atlântica, expostas a poluentes atmosféricos e clima tropical sazonal, foram realizadas por outros autores (Aguiar *et al.,* 2016; Engela 2016), a fim de avaliar respostas antioxidantes nas mesmas espécies. Observou-se que *C. floribundus* apresentou altos níveis de antioxidantes. *C. floribundus* também mostrou ser a espécie mais eficiente em termos de tolerância ao estresse oxidativo do que as espécies não pioneiras (Aguiar *et al.,* 2016). Os níveis foliares desses compostos tenderam a aumentar em resposta a aumentos de radiação solar,

[3] *Funtional group* ou *plant funtional types* – são agrupamentos não filogenéticos de espécies, baseados nas semelhanças no uso de recursos, e resposta às variações ambientais (Duckworth *et al.,* 2000; Gardinier *et al.,* 2016). Portanto, membros do mesmo grupo funcional apresentam um conjunto de *trais* morfológicos, fisiológicos, formas de vida, e/ou histórias de vida similares, ou qualquer outro tipo de função similar, o qual irá depender do objetivo e escala do estudo (Duckworth *et al.,* 2000).

umidade relativa e temperatura do ar e diminuir em resposta a aumento da concentração de $O_3$ e dióxido de nitrogênio ($NO_2$).

Brandão *et al.,* (2018), ao descrever o potencial de tolerância antioxidativa de árvores adultas de nove espécies pioneiras e nove não pioneiras representativas de remanescentes de Floresta Atlântica em São Paulo, também concluíram que as espécies pioneiras tendem a ser mais tolerantes ao estresse oxidativo. Esposito *et al.,* (2018), em amostragens realizadas nos mesmos remanescentes antropizados de floresta estudados por Brandão *et al.,* (2018), constataram que realmente a formação de ROS é mais intensa nas folhas das espécies arbóreas não pioneiras (além de um sistema antioxidante menos eficiente) do que nas folhas das espécies pioneiras. Brandão *et al.* (2018) e Esposito *et al.* (2018) observaram, ainda, que as características bioquímicas das espécies de ambos os grupos funcionais variaram aparentemente em função de efeitos combinados de estressores ambientais de origens natural (como temperatura, radiação solar e umidade relativa do ar) e antrópica (por exemplo, $O_3$ e $NO_2$). Ainda, de acordo com esses estudos, as variações sazonais (períodos seco e úmido), influenciam na variação de características bioquímicas foliares e foram determinantes na separação das espécies avaliadas.

Outro grupo funcional que merece atenção, ao se estudar aclimatação/ adaptação das plantas tropicais e subtropicais frente a distúrbios ambientais, são as lianas[4]. As lianas (principalmente lenhosas e sublenhosas) constituem parte significativa da biomassa das florestas tropicais (DeWalt & Chave 2004; Pérez-Salicrup *et al.,* 2004). Lianas pertencem ao grupo de espécies de plantas heliófilas (crescem sob luz abundante) (Putz 1984; Rocha 2014) e se sustentam em árvores até atingir o dossel da floresta. Apesar das lianas ocorrerem naturalmente em florestas tropicais (Putz 1984;

[4]Lianas – nesta tese, esse termo se refere a plantas não lenhosas (herbáceas) e lenhosas e/ou sublenhosas (como sinônimos) com hábito escalador, conforme definição proposta por Gerwing *et al.,* (2006).

Schnitzer & Bongers 2002), observa-se um aumento significativo da abundância de lianas em florestas perturbadas, principalmente devido ao efeito de borda nas florestas (Campbell *et al.,* 2018). Estas podem prejudicar as árvores, competindo por recursos limitados. Podem diminuir substancialmente as taxas de crescimento e aumentar a taxa de mortalidade das árvores, até mesmo por aumentar a tensão mecânica sob troncos e copas das árvores (Putz 1984; Schnitzer 2005). Como consequência, o domínio de lianas altera a fisionomia da floresta e promove uma redução da capacidade das florestas de sequestrar carbono atmosférico (Phillips *et al.,* 2002; Schnitzer & Bongers 2002; Pivello *et al.,* 2018).

Além da maior disponibilidade de luz em fragmentos florestais ser um fator importante no domínio de lianas em florestas tropicais (Campebell *et al.,* 2018), já foi demonstrado que a abundância de lianas está relacionada negativamente com a precipitação anual e positivamente com a sazonalidade, o que indica seu crescimento mais rápido em relação às árvores sob condições de seca (Schnitzer 2005). Características como heterofilia (Engel *et al.,* 1998), raízes profundas, diâmetro de vasos maiores do que os de espécies arbóreas e arbustivas (o que implica em eficiência de transporte), alta capacidade de armazenamento de água, presença de folhas fotossinteticamente ativas o ano inteiro e crescimento multifocal são vantagens competitivas em relação as árvores, principalmente durante a estação seca (Schnitzer 2005; Jacobsen *et al.,* 2012; Amorim *et al.,* 2018).

## 1.3. Apresentação da Tese

Em síntese, destacamos nos itens anteriores desta introdução geral que as características morfo-anatômicas foliares funcionais e os padrões de respostas morfo-anatomicas podem ser usados para indicar o nível de aclimatação/adaptação de plantas a múltiplos fatores de estresse ambiental, entre climáticos (por exemplo: temperatura e radiação) e/ou poluição atmosférica (poluentes gasosos e material particulado). Destacamos, ainda, a dificuldade de se estudar os efeitos dos estressores ambientais nas florestas tropicais e subtropicais, devido à sua alta biodiversidade, e destacamos que o estudo de espécies representativas de diferentes grupos funcionais pode ser uma estratégia importante para entender a plasticidade de respostas encontradas nessas florestas, aumentando a relevância ecológica dos resultados.

As três etapas experimentais realizadas foram planejadas considerando tal base conceitual. E os resultados obtidos foram organizados e discutidos nos próximos três capítulos. Estes estão escritos em inglês e formatados de acordo com as normas do periódico para o qual foram submetidos. No caso do último capítulo, o artigo já foi publicado (DOI: https://doi.org/10.1016/j.scitotenv.2018.11.425).

O primeiro capítulo trata do uso das características morfo-anatomicas foliares para indicar o potencial de tolerância de 22 espécies arbóreas nativas (pioneiras *vs* não pioneiras) coletadas em quatro fragmentos de Mata Atlântica do sudeste brasileiro.

O segundo capítulo trata das alterações estruturais nas lâminas foliares de espécies arbóreas, entre as relacionadas no primeiro capítulo, com diferentes potenciais de tolerância ao estresse oxidativo provocados por fatores ambientais.

O terceiro capítulo trata da alta capacidade de lianas (outro grupo funcional) em tolerar o estresse oxidativo provocado pelo ozônio troposférico ($O_3$), em sistema FACE (*Free-Air Controlled Exposure Ozone System*).

Finalizamos a Tese apresentando as conclusões e as considerações gerais dos resultados dos três capítulos.

### 1.4. Hipóteses e Objetivos Gerais

Assim, com base na contextualização teórica apresentada, as seguintes hipóteses foram testadas em cada capítulo:

Capítulo 1

H1: Espécies arbóreas pioneiras possuem características morfo-anatômicas que restringem ou evitam os efeitos do estresse oxidativo causados por fatores de estresse naturais (por exemplo, alta radiação solar e déficit de pressão de vapor) e antrópicos (por exemplo, poluentes gasosos e particulados e mudanças climáticas), e, portanto são mais tolerantes que as espécies arbóreas não pioneiras.

H2: Estas características morfo-anatômicas são mais evidentes em espécies arbóreas de remanescentes florestais expostas a condições ambientais mais extremas, como o clima tropical sazonal definido por períodos secos e úmidos bem marcados e altos níveis de poluentes atmosféricos emitidos por fontes urbanas, industriais e agrícolas.

A fim de testar estas hipóteses, objetivou-se:

(i) Identificar, descrever e quantificar as características morfo-anatômicas foliares de espécies arbóreas nativas pioneiras e não pioneiras de remanescentes de Mata Atlântica, diferenciadas por suas características climáticas naturais e proximidade com fontes de emissão de poluentes atmosféricos;

(ii) Agrupar as espécies de acordo com suas características morfo-anatômicas, identificando as possíveis causas para as associações de espécies;

(iii) Reconhecer grupos de gêneros / famílias com potencial de tolerância para sobreviver sob condições ambientais poluídas.

Capitulo 2

H3. Espécies com menor potencial de tolerância a estresses ambientais (variações e anormalidades climáticas e/ou poluentes atmosféricos) apresentam uma maior variedade de marcadores microscópicos em suas lâminas foliares aparentemente saudáveis em resposta ao estresse oxidativo do que espécies com maior potencial de tolerância.

A fim de testar esta hipótese, objetivou-se:

(i) Buscar marcadores microscópicos estruturais em três grupos de espécies com potencial distinto de tolerância ao estresse oxidativo por estressores ambientais (espécies tolerantes, intermediárias e sensíveis);

(ii) Validar o nível de tolerância ao estresse oxidativo em cada grupo com base em marcadores microscópicos.

Capitulo 3

H4. *Passiflora edulis* (espécie de liana) tem uma alta capacidade de adaptação para tolerar o estresse oxidativo causado pela exposição ao ozônio, podendo dominar áreas perturbadas da Mata Atlântica.

A fim de testar esta hipótese, objetivou-se:

(i) Avaliar sintomas visíveis induzidos por ozônio e respostas anatômicas ao estresse oxidativo em *P. edulis* expostas ao Ozônio em sistema FACE (*Free-Air Controlled Exposure Ozone System*), buscando identificar alguns mecanismos de tolerância ou sensibilidade da espécie quando expostas a este poluente. Além disso, respostas fisiológicas e bioquímicas foram avaliadas em conjunto por autores colaboradores para analisar de forma mais abrangente tais mecanimos.

## 1.5. Referências Bibliográficas


Aguiar-Silva, C., Brandão, S.E., Domingos, M., Bulbovas, P. 2016. Antioxidant responses of Atlantic Forest native tree species as indicators of increasing tolerance to oxidative stress when they are exposed to air pollutants and seasonal tropical climate. Ecological Indicators 63: 154–164.

Amorim, T.A., Nunes-Fretiras, A.F., Rosado, B.H.P. 2018. Revisiting the hypothesis for increasing liana abundance in seasonal forest: a theoretical review. Plant Soil 430: 1–6.

Björkman, O. 1981. Responses to different quantum flux densities. In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (Eds.), Physiological Plant Ecology. I: Responses to the Physical Environment. Encyclopedia of Plant Physiology. New Series, v. 12A. Springer-Verlag, Berlin, pp. 57–107.

Blokhina O., Virolainen E, Kurt V.F. 2003. Antioxidants, oxidative damage and oxygen Deprivation stress: a review. Annals of Botany 91: 179–94.

Brandão, S.E., Bulbovas, P., Lima, M.E., Domingos, M. 2017. Biochemical leaf traits as indicators of tolerance potencial in tree species from the Brazilian Atlantic Forest against oxidative environmental stressors. Science Total Environmental 575: 406–417.

Bussotti, F. 2008. Functional leaf traits, plant communities and acclimation processes in relation to oxidative stress in trees: a critical overview. Global Change Biology 14: 2727–2739.

Bussotti, F., Agati, G., Desotgiu, R., Matteini, P., Tani, C. 2005b. Ozone foliar symptoms in woody plant species assessed with ultrastructural and fluorescence analysis. New Phytologist 166: 941–955.

Bussotti, F., Pancrazi, M., Matteucci, G., Gerosa, G. 2005. Leaf morphology and chemistry in *Fagus sylvatica* L. (beech) trees as affected by site factors and ozone: results from Level II permanent monitoring plots in Italy. Tree Physiology 25, 211–219.

Bussotti, F. & Pollastrini, M. 2015. Evaluation of leaf features in forest trees: Methods, techniques, obtainable information and limits. Ecological Indicators 52, 219–230.

Calatayud, V., Cerveró, J., Calvo, E., García-Breijo, F.J., Reig-Armiñana, J., Sanz, M.J. 2011. Responses of evergreen and deciduous *Quercus* species to enhanced ozone levels. Environmental Pollution 159: 55–63.

Campbell, M.J., Edwards, W., Magrach, A., Alamgir, M., Porolak, G., Mohandass, D., Laurance, W.F. 2018. Edge disturbance drives liana abundance increase and alteration of liana–host tree interactions in tropical forest fragments. Ecology and Evolution DOI: 10.1002/ece3.3959.

Chen, X., Zhou, Z., Teng, M., Wang, P., Zhou, L. 2015. Accumulation of three different sizes of particulate matter on plant leaf surfaces: effect on leaf traits. Archives of Biological Sciences 67: 1257–1267.

Cheng, F.Y., Burkey, K.O., Robinson, J.M., Booker, FL. 2007. Leaf extracellular ascorbate in relation to $O_3$ tolerance of two soybean cultivars. Environmental Pollution 150: 355–362.

DeWalt, S.J. & Chave, J. 2004. Structure and Biomass of Four lowland Neotropical

Forests. Biotropica 36: 7–19.

Díaz, S., Purvis, A., Cornelissen, J.H.C., Mace, G.M., Donoghue, M.J., Ewers, R.M., Jordano, P., Pearse, W.D. 2013. Functional traits, the phylogeny of function, and ecosystem service vulnerability. Ecology and Evolution 3: 2958–2975.

Domingos, M., Klumpp, A., Klumpp, G. 2003. Disturbances to the Atlantic Rain Forest in Southeast Brazil In: In air pollution impacts on vegetation in developing countries.1[a]. ed. London: Imperial College Press, pp. 287–308.

Domingos, M., Bulbovas, P., Camargo, C.Z.S., Aguiar-Silva, C., Brandão, S.E., Dafré-Martinelli, M., Dias, A.P.L., Engela, M.R.G.S., Gagliano, J., Moura, B.B., Alves, E.S., Rinaldi, M.C.S., Gomes, E.P.C., Furlan, C.M., Figueiredo, A.M.G., 2015. Searching for native tree species and respective potential biomarkers for future assessment of pollution effects on the highly diverse Atlantic Forest in SE-Brazil. Environmental Pollution 202: 85–95.

Duckworth, J.C., Kent, M., Ramsay, P.M. 2000. Plant functional types: an alternative to taxonomic plant community description in biogeography? Progress in Physical Geography 24: 515–542.

Engel, V.L., Fonseca, R.C.B., Oliveira, R.E. 1998. Ecologia de lianas e o manejo de fragmentos florestais. Série Técnica IPEF 12: 43–64.

Engela, M.R.G.S. 2016. Variações no conteúdo e na composição de carboidratos solúveis e de compostos fenólicos em espécies arbóreas de Floresta Estacional Semidecidual em resposta a poluentes aéreos. Dissertação de Mestrado, Instituto de Botânica de São Paulo.

Espostio, M.P., Nakazato, R.K., Pedroso, A.N.V., Lima, M.E.L., Figueiredo, M.A., Diniz, A.P., Kozovits, A.R., Domingos, M. 2018. Oxidant-antioxidant balance and tolerance against oxidative stress in pioneer and non-pioneer tree species from the remaining Atlantic Forest. Science of the Total Environment 625: 382–393.

Faoro, F. & Iriti, M. 2009. Plant cell death and cellular alterations induced by ozone: Key studies in Mediterranean conditions. Environmental Pollution 157: 1470–1477.

Ferdinand, J.A., Fredericksen, T.S., Kouterick, K.B., Skelly, J.M. 2000. Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. Environmental Pollution 108: 297–302.

Fernandes, F.F., Cardoso-Gustavson, P., Alves, E.S. 2016. Synergism between ozone and light stress: structural responses of polyphenols in a woody Brazilian species. Chemosphere 155: 573–582.

Fernandes, F.F., Esposito, M.P., Engela, M. R. G. da S., Cardoso-Gustavson, P., Furlan, C.M., Hoshika, Y., Carrari, E., Magni, G., Domingos , M., Paoletti, E. 2019. The passion fruit liana (*Passiflora edulis* Sims, Passifloraceae) is tolerant to ozone. Science of the Total Ennvironment 656, 1091–1101.

Fiscus, E.L., Booker, F.L., Burkey, K.O. 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant Cell Environmental 28: 997–1011.

Foyer, C.H. & Shigeoka, S. 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Physiologia Plantarum 155, 93–100.

Fuhrer, J. & Booker, F. 2003. Ecological issues related to ozone: agricultural issues. Environmental International 29: 141–54.

Garnier, E., Navas, M.L., Grigulis, K. 2015. Plant Functional Diversity Organism traits, community structure, and ecosystem properties-Oxford University Press, United Kingdom.

Gerosa, G., Marzuoli, R., Rossini, M., Panigada, C., Meroni, M., Colombo, R., Faoro, F., Iriti, M. 2009. A flux-based assessment of the effects of ozone on foliar injury, photosynthesis, and yield of bean (*Phaseolus vulgaris* L. cv. Borlotto Nano Lingua di Fuoco) in open-top chambers. Environmental Pollution 157: 1727–1736.

Gerwing, J.J. Schnitzer, S.A., Burnham, R.J., Bongers, F., Chave, J., DeWalt, S.J., Ewango, C.E.N., Foster, R., Kenfack, D., Mart´ınez-Ramos, M., Parren, M., Parthasarathy, N., Perez-Salicrup, D.R., Putz, F.E., Thomas, D.W. 2006. A Standard Protocol for Liana Censuses. Biotropica, 38: 256–261.

Gratão, P.L., Monteiro, C.C., Carvalho, R.F., Tezotto, T., Piotto, F.A., Peres, L.E.P., Azevedo, R.A. 2012. Biochemical dissection of *diageotropica* and *Never ripe* tomato mutants to Cd-stressful conditions. Plant Physiology Biochemestry 56: 79–96.

Gravano, E., Bussotti, F., Strasser, R.J., Schaub, M., Novak, K., Skelly, J., Tani, C. 2004. Ozone symptoms in leaves of woody plants in open-top chambers: ultrastructural and physiological characteristics. Physiologia Plantarum 121: 620–633.

Guerrero, C.C., Günthardt-Goerg, M.S., Vollenweider, P. 2013. Foliar Symptoms Triggered by Ozone Stress in Irrigated Holm Oaks from the City of Madrid, Spain PLoS ONE 8, e69171.

Günthardt-Goerg, M.S., McQuattie, C.J., Maurer, S., Frey, B. 2000. Visible and microscopic injury in leaves of five deciduous tree species related to current critical ozone levels. Environmental Pollution 109: 489–500.

Günthardt-Goerg, M.S., McQuattie, C.J., Scheidegger, C., Rhiner, C., Matyssek, R. 1997. Ozone-induced cytochemical and ultrastructural changes in leaf mesophyll cell. Canadian Journal of Forest Research 27: 453–463.

Gurevitch, J., Scheiner, S.M., Fox, A.G. 2009. Ecologia vegetal. 2 ed. Artmed Editora, Porto Alegre, pp. 205– 207.

Jacobsen, A.L., Pratt, R.B., Tobin, M.F., Hacke, U.G., Ewers, F. W. 2012. A global analysis of xylem vessel length on woody plants. American Journal of Botany. v.99, n. 10, pp. 1583– 1591.

Jochner, S., Markevych, I., Beck, I., Traidl-Hoffmann, C., Heinrich, J., Menzel, A. 2015. The effects of short- and long-term air pollutants on plant phenology and leaf characteristics. Environmental Pollution 206: 382–389.

Kivimäenpää, M., Riikonen, J., Sutinen, S., Holopainen, T. 2014. Cell structural changes in the mesophyll of Norway spruce needles by elevated ozone and elevated temperature in open-field exposure during cold acclimation. Tree Physiology 34: 389–403.

Kivimäenpää, M., Sutinen, S., Karlsson, P.E., Selldén, G. 2003. Cell Structural Changes in the Needles of Norway Spruce Exposed to Long-term Ozone and Drought.

Annals of Botany 92: 779–793.

Lusk, C.H, Reich, P.B.; Montgomery, R.A., Ackerly, D.D.; Cavender-Bares, J. 2008. Why are evergreen leaves so contrary about shade? Trends in Ecology & Evolution 23: 299–303.

Matyssek, R., Wieserb, G., Calfapietra, C., de Vriesd, W., Dizengremelf, P., Ernst, D., Jolivet, Y., Mikkelsen, T.N., Mohren, G.M.J., Le Thiec, D., Tuovinen, J.P., Weatherall, A., Paoletti, E. 2012. Forests under climate change and air pollution: gaps in understanding and future directions for research. Environmental Pollution 160: 57–65.

Morgan, P.B., Mies, T.A., Bollero, G.A., Nelson, R.L., Long, S.P. 2006. Season-long elevation of ozone concentration to projected 2050 levels under fully open-air conditions substantially decreases the growth and production of soybean. New Phytologist 170: 333–43.

Moura, B.B. & Alves, E.S. 2014. Climatic factors influence leaf structure and thereby affect the ozone sensitivity of *Ipomoea nil* 'Scarlet O'Hara'. Environmental Pollution 194: 11–16.

Moura, B.B., Alves, E.S., Marabesi, M.A., de Souza, S.R., Schaub, M., Vollenweider, P. 2018. Ozone affects leaf physiology and causes injury to foliage of native tree species from the tropical Atlantic Forest of southern Brazil. Science of the Total Environment, 610–611: 912–925.

Nakazato, R.K., Esposito, M.P., Cardoso-Gustavson, P., Bulbovas, P., Pedroso, A.N.V., Assis, P.I.L.S., Domingos, M. 2018. Efficiency of biomonitoring methods applying tropical bioindicator plants for assessing the phytoxicity of the air pollutants in SE, Brazil. Environmental Science and Pollution Research 25:19323–19337.

Nock, C.A., Vogt, R.J., Beisner, B.E. 2016. Functional Traits. In: eLS. John Wiley & Sons, Ltd: Chichester DOI: 10.1002/9780470015902.a0026282

Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M. 2010. Heavy metals, occurrence and toxicity for plants: a review. Environmental Chemistry Letters 8:199–216.

Oksanen, E., Häikiö, E., Sober, J., Karnosky, D.F. 2003. Ozone-induced $H_2O_2$ accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity New Phytologist 161:791–799.

Oksanen, E., Pandey, V., Pandey, A.K., Keski-Saari, S., Kontunen-Soppela, S., Sharma, C. 2013. Impacts of increasing ozone on Indian plants Environmental Pollution 177: 189–200.

Pääkkönen, E., Holopainen, T., Kärenlampi, L. 1997. Variation in ozone sensitivity among clones of *Betula pendula* and *Betula pubescens.* Environmental Pollution 95: 37–44.

Paoletti, E., Contran, N., Bernasconi, P., Günthardt-Goerg, M.S., Vollenweider, P. 2009. Structural and physiological responses to ozone in Manna ash (*Fraxinus ornus* L.) leaves of seedlings and mature trees under controlled and ambient conditions. Science of the Total Environmental 407: 1631–1643.

Paoletti, E., Seufert, G., Rocca, G.D., Thomsen, H. 2007. Photosynthetic responses to elevated $CO_2$ and $O_3$ in *Quercus ilex* leaves at a natural $CO_2$ spring. Environmental Pollution 147: 516–524.

Pedroso, A.N.V. & Alves, E.S. 2015. Temporal dynamics of the cellular events in tobacco leaves exposed in São Paulo, Brazil, indicate oxidative stress by ozone. Environmental Science and Pollution Research 22: 6535–45.

Pedroso, A.N.V., Bussotti, F., Papini, A., Tani, C., Domingos, M. 2016. Pollution emissions from a petrochemical complex and other environmental stressors induce structural and ultrastructural damage in leaves of a biosensor tree species from the Atlantic Rain Forest. Ecological Indicators 67: 215–226.

Pérez-Salicrup, D.R., Schnitzer, S.A., Putz, F.E. 2004. The community ecology and management of lianas. Forest Ecology Management 190: 1–2.

Phillips, O.L., Martinez, R.V., Arroyo, L., Baker, T.R., Killeen, T., Lewis, S.L., Malhi, Y., Mendoza, A.M., Neill, D., Vargas, P.N., Alexiades, M., Cerón, C., Di Fiore, A., Erwin, T., Jardim, A., Palacios, W., Saldias, M., Vinceti, B. 2002. Increasing dominance of large lianas in Amazonian forests. Nature 418: 770–774.

Pivello, V.R., Vieirab, M.V., Grombone-Guaratinic, M.T., Matos, D.M.S. 2018. Thinking about super-dominant populations of native species – examples from Brazil. Perspectives Ecology Conservation. 16: 74–82.

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I., Villar, J.R. 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytology 182: 565–588.

Putz, F.E. 1984. the natural history of lianas on Barro Colorado Island, Panama Ecology, 65(6), 1984, pp. 1713–1724.

Rocha, E.X. 2014. Composição de espécies de lianas e sua resposta ao corte em um fragmento de floresta estacional semidecidual, Araras, SP. Dissertação de metrado apresentada ao Programa de Pós-graduação em Agricultura da Universidade Federal de São Carlos, Centro de Ciências Agrárias.

Roschina, V.V., Roschina, V.D. 2003. Ozone and plant cell. Dordrecht, Kluwer Academic Publishers.

Schnitzer, S.A. & Bongers, F. 2002. The ecology of lianas and their role in forests. Trends in Ecology & Evolution 17: 223–230.

Schnitzer, S.A. 2005. A mechanistic explanation for global patterns of liana abundance and distribution. The American Naturalist 166: 262–276.

Schraudner, M., Moeder, W., Wiese, C., Camp, W.V., Inze, D., Langebartels, C., Sandermann, Jr.H. 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. The Plant Journal 1: 235–245.

Shahid, M., Dumat, C., Khalid, S., Schreck, E., Xiong, T., Niazi, N.K. 2012. Foliar heavy metal uptake, toxicity and detoxification in plants: A comparison of foliar and root metal uptake. Journal of Hazardous Materials 325: 36–58.

Ueda, Y., Uehara, N., Sasaki, H., Kobayashi, K., Yamakawa, T. 2013. Impacts of acute ozone stress on superoxide dismutase (SOD) expression and reactive oxygen species (ROS) formation in rice leaves. Plant Physiology Biochemestry 70: 396–402.

Violle, C., Navas, M.L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., Garnier, E., 2007. Let the concept of trait be functional! Oikos 116: 882–892.

Vollenweider, P., Cosio, C., Günthardt-Goerg, M. S., Keller, C. 2006. Localization and

effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.): Part II Microlocalization and cellular effects of cadmium. Environmental and Experimental Botany 58: 25–40.

Vollenweider, P., Fenn, M.E., Menard, T., Günthardt-Goerg, M., Bytnerowicz, A. 2013. Structural injury underlying mottling in ponderosa pine needles exposed to ambient ozone concentrations in the San Bernardino Mountains near Los Angeles, California. Trees 27: 895–911.

Vollenweider, P., Ottiger, M., Günthardt-Goerg, M.S. 2003. Validation of leaf ozone symptoms in natural vegetation using microscopical methods. Environmental Pollution 124:101–118.

Xu, F.; Guo, W., Xu, W., Wei, Y., Wang, R. 2009. Leaf morphology correlates with water and light availability: What consequences for simple and compound leaves? Progress in Natural Science 19: 1789–1798.

Yamasaki, H., Sakihama, Y., lkehara, N. 1997. Flavonoid-Peroxidase Reaction as a Detoxification Mechanism of Plant Cells against $H_2O_2$. Plant Physiology 115: 1405–1412.

Zampieri, M.C.T., Sarkis, J.E.S., Pestana, R.C.B., Tavares, A.R., Gladys F.A., Melo-de-Pinna. 2013. Characterization of *Tibouchina granulosa* (Desr.) Cong. (Melastomataceae) as a biomonitor of air pollution and quantification of particulate matter adsorbed by leaves. Ecological Engineering 61: 316–327.

Capítulo 1

# Morpho-anatomical leaf traits indicate the strategies of tree species to tolerante environmental stressors in the remaining Brazilian Atlantic Forest[5]

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[5]Capítulo a ser submetido ao periódico Ecological Indicators (QUALIS A2; IF 4.4).

**Abstract**

Early studies showed that pioneer tree species have physiological and biochemical leaf traits that confer a higher tolerance against oxidative stress than non-pioneer species in high diverse tropical forests. However, morpho-anatomical leaf traits in particular are good predictors of acclimation or susceptibility levels of forest species to environmental stress, and may contribute to increase the plasticity of these functional groups in the remaining Brazilian Atlantic forest. Here we connect these previous studies by evaluating the mopho-anatomical aspects of pioneer and non-pioneer tree species. We hypothesized that (1) pioneer tree species also have morpho-anatomical traits that restrict or avoid the effects of oxidative pressure (gaseous and particulate pollutants and climatic changes); (2) these morpho-anatomical traits are more evident in tree species from forest remnants exposed to more extreme environmental conditions. These hypothesis were tested in species from a seasonal tropical climate defined by well-marked dry and wet periods and high levels of air pollutants emitted by urban, industrial and agricultural sources. We confirmed the first hypothesis. Pioneer tree species produce leaves with higher leaf mass area (LMA) and leaf density (LD), palisade parenchyma thickness (PP), abaxial stomatal density (SD) and abaxial trichomes density (TD). Conversely, non-pioneer species produce opposite morpho-anatomical traits and are less adapted and acclimated to the oxidative stress imposed by environmental conditions. The second hypothesis was tested and rejected. The absence of spatial variation on morpho-anatomical leaf traits may suggest the influence of the evolutionary history of the evaluated species.

**Keywords:** environmental stressors, leaf anatomy, leaf morphology, phylogenetic aspects, tropical tree species.

## 1. Introduction

The tolerance or sensitivity of plants to the environmental pressures is determined by their phenotypic plasticity in response to the oxidative stress (Bussotti, 2008; Moura et al., 2014a; Bussotti and Polastrini, 2015). A high phenotypic plasticity allows the species to survive under a wide variety of environmental conditions, reducing the risk of extinction due to the environmental changes (Bussoti and Polastrini, 2015).

The phenotypic plasticity of plant species is determined by their functional traits, including phenological, morpho-anatomical, physiological, biochemical, among other characteristics, which will influence altogether their performance or fitness in the natural environment (Nock et al., 2016). The physiological and biochemical traits have already been considered in studies to assess the healthy status of tree species of the remaining Atlantic Forest in SE Brazil, which has been the target of disturbances caused by multiple environmental stressors (Domingos et al., 2003, 2015; Brandão et al., 2017; Esposito et al., 2018). Among the native tree species evaluated in these studies, *Tibouchina pulchra* (Melastomataceae) and *Croton floribundus* (Euphorbiaceae) have physiological and biochemical traits that enable them to tolerate the environmental oxidative stress, meanwhile *Astronium graveolens* (Anacardiaceae) was characterized as intermediate tolerant and *Piptadenia gonoacantha* (Fabaceae) as sensitive (Aguiar-Silva et al., 2016; Esposito et al., 2016, 2018; Nakazato et al., 2018).

Leaves play a key role in the acclimation and long-term adaptation of plants to the environment (Bussotti and Pollastrini, 2015; Tian et al., 2016). Morpho-anatomical leaf traits in particular are good predictors of how susceptible a species may be to environmental stressors (Bussotti, 2008; Bussotti and Pollastrini, 2015; Domingos et al., 2015; Li et al., 2016). Some characteristics, e.g. palisade and spongy parenchyma (photosynthetically active tissues) thickness, airspace fraction, vein diameter, stomatal

density (de la Riva et al., 2016; Tian et al., 2016; John, 2017), epicuticular wax (Koch and Ensikat, 2008), presence, abundance and distribution of trichomes (Domingos et al., 2015), foliar density, and leaf area are among the features described as suitable functional leaf traits for such purposes. These characteristics may vary among species and plant communities in response to different environmental conditions and also among functional groups of plants, e.g. sun/shade species or pioneer/non-pioneer species (Poorter et al., 2009 Violle et al., 2007; Díaz et al., 2013; Bussotti and Pollastrini, 2015; Nock et al., 2016). Moreover, we may not disregard the fact that the morpho-anatomical leaf traits can be conserved phylogenetically throughout the taxon evolution, explaining the similarity of leaf traits observed in certain genera and families in some studies (Poorter and Bongers, 2006; Poorter et al., 2009).

However, it would be unfeasible to discuss the potential acclimation or susceptibility to environmental changes at the ecosystem level in high diverse tropical forests, such as the Brazilian Atlantic forest. The laborious measurements of several functional traits would be restricted to a few number of plant species (Esquivel-Muelbert et al., 2017), turning the study a challenging task. The choice of 'soft traits' (here referred to morpho-anatomical traits) is a rational option, since they are easily and quickly quantified, allowing the increase in the number of species included in the study (Nock et al., 2016), but not necessarily the ecological relevance of the results.

The evaluation of the level of acclimation or susceptibility to environmental stress in forest species showing contrasting ecological functions in the ecosystem, such as those defined by the successional process is an alternative to surpass this sampling difficulty. Brandão et al. (2017) and Esposito et al. (2018) identified the tolerance potential of the pioneer and non-pioneers tree species based on the variations of the biochemical leaf traits in the remaining Atlantic Forest under a climatic and atmospheric

pollution gradient. The oscillations in these leaf traits of tree species included in both functional groups were explained by combined effects of climatic conditions and air pollutants, suggesting possible acclimation responses of both groups of species to environmental stressors. However, the pioneer species have physiological and biochemical leaf traits that seem to confer a higher tolerance against oxidative stress than those of non-pioneer trees. Therefore, the question that arises is whether morpho-anatomical traits would contribute to increase the plasticity of these functional groups of tree species from the remaining Brazilian Atlantic forest, enhancing their potential tolerance against environmental stressors. The present field study was planned based on this question. We assumed the following hypotheses: (1) pioneer tree species also have morpho-anatomical traits, that restrict or avoid the effects of oxidative stress posed by natural (e.g. high solar radiation and vapor pressure deficit) and anthropic (e.g. gaseous and particulate pollutants and climatic changes) stressors than non-pioneer species; (2) these morpho-anatomical traits are more evident in tree species from forest remnants exposed to more extreme environmental conditions, such as the seasonal tropical climate defined by well-marked dry and wet periods and high levels of air pollutants emitted by urban, industrial and agricultural sources.

In order to answer the question proposed and evaluate the veracity of these hypotheses, we: (i) identified, described and quantified the morpho-anatomical leaf traits of pioneer and non-pioneer trees species from Atlantic forest remnants distinguished by their natural climatic characteristics and proximity to emission sources of air pollution; (ii) clustered the species according to their morpho-anatomical leaf traits, identifying the possible causes for the species associations; and (iii) recognized groups of species/genera/families with high potential tolerance to survive under stressing environmental conditions in the tropics.

This approach over species of the Atlantic Forest remnants may further shed light on the mechanisms that governs the distribution and abundance of species in a disturbed tropical environment.

## 2. Material and methods

### 2.1. Species selection and field sampling procedures

The study was developed in four remnants of Atlantic Forest in Southeast Brazil, which differ in forest physiognomy and plant species composition due to their natural climatic characteristics and proximity to emission sources of pollution (Table 1). Three of them are included in conservation units located in the state of São Paulo (Municipal Park Paranapiacaba, MPP; State Park Fontes do Ipiranga, PEFI and an ecological area of relevant interest Mata de Santa Genebra, MSG), and the last in the state of Minas Gerais (State Park Itacolomi, PEI). The MPP forest site is farther from pollution sources than the other forest remnants. The PEFI, MSG and PEI forest sites are next to urban, urban/agricultural/industrial and mining/industrial pollution sources, respectively (see Brandão et al., 2017 and Esposito et al., 2018, for more details).

**Table 1.** Characteristics of the remnants of Atlantic Forest in Southeast Brazil included in this study. Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI).

| | MPP | PEFI | MSG | PEI |
|---|---|---|---|---|
| Area | 400 ha | 540 ha | 252 ha | 7500 ha |
| Geographical coordinates | 23°46′41″S 46°18′16″W | 23°40′18″S 46°38′00″W | 22°49′22.65″S 47°06′17.38″W | 20°25′58.552″S 43°30′42.7054″W |
| Mean altitude | 890 m | 770 m | 670 m | 1200 m |
| Climate (Koeppen classification) | Cfb (humid subtropical, without dry season and temperate summer) | Cwb (humid subtropical, with dry winter and temperate summer) | Cwa (humid subtropical, with dry winter and hot summer) | Cwb (humid subtropical, with dry winter and temperate summer) |
| Mean annual rainfall | 3300 mm | 1500 mm | 1400 mm | 1800 mm |
| Air pollutants sources | less afected by pollution sources than the other forest remnants | urban | urban/agricultural /industrial | mining/industrial pollution |

The native tree species were selected based on their representativeness in each forest remnant, on their functional group and on their taxonomic similarity (the same genera/family) whenever possible. This information were obtained from previous floristic surveys (Guaratini et al., 2008; Lima et al., 2011; Pedreira and Sousa, 2011; Tanus et al., 2012). Three pioneer species and three to four non-pioneer species were selected in each remnant of Atlantic Forest – see the list of tree species in Table 2 and in Tables S1 and S2 (supplementary material).

A single leaf collection in each forest site was performed during January to August/2016. Approx. four to five branches containing sunny leaves were collected

from four specimens per species in each forest remnant.

### 2.2. Measurement of leaf traits

Fully expanded leaves from the middle portion of the branches of each specimen were excised for the analysis of morphological (Table 2) and anatomical traits (Table 2), avoiding the sampling of senescent leaves and those with visible symptoms caused by any biotic or abiotic agent. The morphological analyses were performed in fresh leaves immediately after the collection and the anatomical analyses after specific leaf preparations as described below.

**Table 2.** Assessement the funtional leaf traits.

| **Funtional leaf traits** | **Abbreviation** | **Determination** | **Unit** |
|---|---|---|---|
| **Morphological traits*** | | | |
| Leaf area | LA | Direct measurement | $cm^2$ |
| Leaf mass per area | LMA | LA / dry weight | mg $cm^{-2}$ |
| Leaf density | LD | dry weight (LA x LT) | mg $cm^{-3}$ |
| Dry matter concentration | DMC | dry weight / (LA × LT) | mg $cm^{-3}$ |
| Relative water content | RWC | [(fresh weight − dry weight) / (saturated weight – dry weight)] × 100 | % |
| **Anatomical traits** | | | |
| Palisade parenchyma thickness | PP | Direct measurement | μm |
| Spongy parenchyma thickness | SP | Direct measurement | μm |

| | | | |
|---|---|---|---|
| Intercellular space | IS | Number of pixels that occupies the leaf blade/ number of pixels that occupies the inter cellular space) x 100 | % |
| Stomatal density | SD | The number of stomata per unit area | $mm^2$ |
| Trichomes density | TD | The number of trichomes per unit area | $mm^2$ |

*Based on Bussotti (2008); Bussotti and Pollastrini (2015).

**2.2.1. Morphological leaf traits (see Table 2)**

Leaf area (LA, $cm^2$) was estimated in a total of ten leaves of each tree per species using a scanner image (Lexmark MX810de) and ImageJ (1.46 r). Ten to thirty additional leaves per specimen of each species were used to measure the other morphological leaf traits. One disc (area: from 3.036 $cm^2$ to 4 $cm^2$) was cut from the median portion of each leaf blade, avoiding the midrib. The fresh weight (mg) was measured, and the discs were then incubated overnight in deionized water to obtain the saturated weight (Barr and Weatherley, 1962). Afterwards, these same leaf discs were dried under 60 °C (until reaching constant weight) to obtain leaf dry weight. The leaf area and dry weight of *Piptadenia gonocantha* trees were obtained in a parallel study (Domingos et al., 2015) developed in the same forest remnant included in the present research (MSG) because their leaflets are very small and fall easily, turning impossible the measurement of the saturated weight on leaf discs.

**2.2.2. Anatomical leaf traits (see Table 2)**

Four leaves per tree were used for measuring anatomical leaf traits. Fragments of the leaf blade were fixed in 2.5 % glutaraldehyde buffered at pH 7.0 with 0.067 M

Sorensen phosphate buffer, and placed under vacuum before storing at 4 °C. The fixed samples were included in Technovit 7100 historesin and semi-thin sectioned to 3 μm thickness in rotary microtome Leica (RM2245). The slides were stained with toluidine blue/p-phenylenediamine for metachromasia and lipid detection, respectively (Feder and O'Brien, 1968/Kivimäenpää et al., 2004), and the images captured in an Olympus BX53 microscope equipped with an image capture system (Image Pro-express software 6.3) for quantitative analysis the of leaf tissues.

The thickness of the leaf (from upper to lower epidermis; LT, μm), palisade parenchyma (PP, μm) and spongy parenchyma (SP, μm) were measured in transverse sections of the leaf blade using the software ImageJ (1.46 r). Two measurements were performed in each image, avoiding the vascular regions. Eight measurements per tree were acquired, totalizing 32 for each tree species. In addition, the percentage of intercellular spaces (IS, %) was measured using Adobe Photoshop CC (14.2). One measurement was made in each image, totalizing four estimates per tree (16 for each tree species).

Three dried leaves of each species were hydrated and diaphonized using 10% sodium hydroxide and 20% hypochlorite solution. Images from adaxial and abaxial leaf surfaces were captured to calculate stomata density (SD, $N/mm^2$ - was counted from the images at a magnification of 40×) and trichomes density (TD, $N/mm^2$ - was counted from the images at a magnification of 20×) using the software ImageJ (1.46 r). The trichomes present on the veins (except the midrib) were also considered in the quantification.

For surface microanalysis, fragments of the leaf median region were fixed in 2.5 % glutaraldehyde buffered at pH 7.0 with 0.067 M Sorensen phosphate buffer, dehydrated in alcohol and dried in a critical point dryer (Leica EM CPD300). Samples

were mounted on stubs, coated with gold in a sputtering system (Leica ACE200) and observed with a SEM FEI Quanta 250 at 10kV for describing the morphology of epicuticular waxes and trichomes of both leaf surfaces of each species. In addition, particulate matter (PM) adhered to the leaf lamina and epidermal structures were also registered. Digital images were edited using Adobe Photoshop version 7.0.

**2.3. Data presentation and statistics**

First, medians ± standard deviations were calculated for each morphological and anatomical leaf trait of the tree species (N= 4 tree per species) in each remnant of Atlantic Forest. One-way ANOVA on ranks (Kruskal Wallis test) followed by a multiple comparison (Dunn's method) were then applied to indicate significant differences in the results obtained for each morphological and anatomical leaf trait between the tree species in each remnant of Atlantic Forest (these results were included Tables S1 andS2 and described in the supplementary material).

Subsequently, mean values ± standard deviations were calculated for pioneer and non-pioneer species (N=13 pioneer and 12 non-pioneer species), independent the location the leaf samples were obtained. Two-way ANOVA was applied to indicate differences between the functional group (pioneer species x non-pioneer species, factor 1) and the sapling sites (factor 2). The leaf traits were $\log_{10}$ or square root transformed, when necessary, to reach the normal distribution and equal variances. The stomata and trichome densities on the adaxial surface were excluded from this data analysis because most species are hipostomatic and have few trichomes on the adaxial surface.

A cluster analysis was finally performed to verify the similarity level among the species considering the following traits: LA, LMA, LD, DMC, LT, PP, SP, IS, SD and TD (the last two, only on the abaxial surface). Each species was associated with its functional group and forest of origin, aiming to verify if the species were grouped

according to the functional group, sampling location or taxonomic proximity. The dendrogram was generated by the Ward method and Euclidean distances. The exclusion of TD and SD on the adaxial surface and RWC was due to the low data variability. The values were all standardized and computed as follows: Std. Score = [(raw score - mean)/Std. deviation]. In addition, principal component analyses (PCA) was performed using the values normalized by $\log_{10}$ in order to investigate which leaf traits better explained the groups of species highlighted by the cluster analyses.

## 3. Results

### 3.1. Quantitative variations in morphological and anatomical leaf traits of tree species

The morphological and anatomical leaf traits differed significantly among the tree species sampled in each site and the differences are detached and described in the supplementary material (Tables S1 and S2).

As indicated by the Two-way ANOVA, some morphological and anatomical leaf traits differed significantly between pioneer and non-pioneer species (factor 1), as highlighted below, but any trait differed significantly among forest remnants (factor 2). In addition, interactions between functional groups and forest remnants were not proved for all traits analyzed, indicating that the differences between pioneer and non-pioneer species, when proved by ANOVA, are valid for all forest remnants (Table 3). Therefore, aiming at simplifying the result presentation, the morphological and anatomical traits of pioneer and non-pioneer species were presented as average values in Table 3, considering data from all forest sites, followed by respective standard deviations.

The pioneer species from all forest sites were characterized by a higher LMA and LD than the non-pioneer species. Species from both functional group did not differ

in relation to the other morphological leaf traits evaluated (Table 3).

Pioneer tree species showed higher values of PP thickness, abaxial SD and abaxial TD than the non-pioneer species. Conversely, the SP thickness was higher in non-pioneer species than in the pioneer species. Species from both functional group did not differ in relation to the other anatomical leaf traits evaluated (Table 3).

**3.2. Clustering the tree species according to their leaf traits**

Cluster analysis separated the species in two distinct groups at the Euclidean linkage distance of 20, according to the similarities of their leaf traits (cluster 1 and cluster 2, Fig. 1A). The cluster 1 was separated into three main subgroups (cluster 1A-C, Fig. 1A) and the cluster 2 into two subgroups of species (cluster 2A and 2B; Fig. 1A) at the distances of 8 and 15 respectively. The PCA summarized 66% of the total variability of the data on the first two axes. The strongest correlation with axis 1 was found for LMA (r = - 0. 91), followed by DMC (r = -0.79), PP (r = -0.72), TD (r = 0.63) and SD (r = 0.60; Fig. 2B), respectively. The strongest correlation with axis 2 was found for SP (r = 0.92), followed by LT (r = 0.88) and LD (r = 0.67), respectively (Table in Fig. 1B). The groups of species formed by cluster analysis were also highlighted by PCA (Fig. 1B).

The cluster 1A joined the trees of six non-pioneer species (*Amaioua intermedia, Guarea macrophylla, Ocotea beulahiae, Ocotea paranapiacabensis, Psychotria suterella* and *Psychotria vellosiana*), which were positioned in the negative side of axis 1 of PCA and were characterized by a low leaf mass area (LMA), dry matter concentration (DMC), palisade thickness (PP), abaxial stomatal density (SD) and abaxial trichome density (TD). The cluster 1B grouped five pioneer (*Alchornea sidifolia, Alchornea triplinervia*, *Croton floribundus, Piptadenia gonoacantha* and *Solanum granulosoleprosum*) and three non-pioneer species (*Astronium graveolens,*

*Eugenia excelsa* and *Machaerium villosum*), which were located in the negative side of axis 2 of PCA. These species were described by a high LD and low LT and SP. The cluster 1C grouped three pioneer (*Eremanthus erytropappus*, *Schinus terebinthifolius* and *Tibouchina pulchra*) and one non-pioneer species (*Eugenia cerasiflora*), which were located next to the intersection between axis 1 and 2 of PCA and had intermediate values of LMA, DMC, PP, SD and TD. The cluster 2A was represented by one pioneer (*Myrsine umbellata*) and two non-pioneer (*Drymis brasiliensis* and *Guarea kunthiana*) trees, positioned in the positive side of axis 2 of PCA, and characterized by a high LT and SP and low LD. The specimens of *Miconia cabucu* (cluster 2B) were located in the negative side of axis 1 and correlated with high LMA, PP, DMC, TD and SD regardless of the sampling site. The species were not clearly grouped according to the forest where they were sampled (Fig.1A-B).

**3.3. Description of the leaf blade surface**

The species differed in the morphology of epicuticular waxes and epidermal cells, and distribution of trichomes. The main differences between the species are included in Table 4 and Fig. 2 and described below – for more details, see Fig. S1 in the supplementary material.

A higher particle deposition was observed on the adaxial surface (Fig. 2A *vs.* J). A smooth cuticle was identified in species with glabrous leaves (*A. intermedia, E. ceraciflora, E. excelsa, O. beulahiae, O. paranapiacabensis*; Table 4; Fig. 2A) and with hairy leaf blades (*A. sidifolia*, *D. brasiliensis*, *E. erytropappus, M. umbellata, T. pulchra* and *S. granulosoleprosum*; Table 4, Fig. 2B-F). A rough cuticle resulting from the ornamentation of epicuticular wax was also identified in species with glabrous leaves (*G. kunthiana* and *S. terebinthifolia*; Table 4), and with hairy leaf blades (*A. triplinervia, A. graveolens, G. macrophylla, P. gonoacantha, P. suterella*; Table 3; Fig.

2G-I). In addition, species that have the cuticle with evident contours (Fig. 2F) and. striations (Fig. 2H) (e.g. *A. intermedia, A. graveolens, Croton floribundus, S. granulosoleprosum*, *G. kunthiana* and *P. suterella)* also presented particle deposition on it. Leaf blades densely covered by trichomes were observed in some tree species (*C. floribundus*, *E. erytropappus*, *M. villosum*, *S. granulosoleprosum* and *T. pulchra*; Table 2; Fig. 2D-F). Particle deposition was also observed over the adaxial surface covered by non-glandular trichomes, as observed in *A. triplinervia*. *C. floribundus, M. villosum* and *S. granulosoleprosum* (Table 4; Fig. 2F-H). Particle deposition was also detected in stomata of *O. beulahiae* and *O. paranapiacabensis*, obstructing the stomatal pore (Fig. 2J).

A smooth cuticle in the abaxial leaf surface was identified in the glabrous leaves of *O. beulahiae* and *O. paranapiacabensis* (Table 4; Fig. 2J), and in the hairy leaf blades of *A. intermedia* and *G. kunthiana* (Table 4). Wax deposition in platelets, granules, striations, and elevations was observed in several species (*A. sidifolia, A. triplinervia, A. graveolens, D. brasiliensis, E. erytropappus, G. macrophylla, M. villosum, M. umbellata, P. gonoacantha, P. suterella, S. terebinthifolius* and *T. pulchra*; Table 4; Fig. 2K-N, P-R). The leaves of *C. floribundus, M. cabucu* and *S. granulosoleprosum* were densely covered by non-glandular trichomes (Table 4; Fig. 2O). Few species were glabrous (*D. brasiliensis* and *E. erytropappus*; Table 4; Fig 2L-M). Fungal hyphae associated with particulate matter were observed on both surfaces of these species (Fig. 2A, 1C-F, 2H and J).

**Table 3**. Means values (± standard deviations) for morphological and anatomical leaf traits of pioneer and non-pioneer tree species sampled in different remnants of Atlantic Forest. LA, leaf area; LMA, leaf mass area; LD, leaf density; DMC, dry matter concentration; RWC, relative water content; LT, leaf thickness; PP, palisade parenchyma thickness; SP, spongy parenchyma thickness; IS, intercellular space; SD, abaxial stomatal density; TD, abaxial trichomes density. Two-way ANOVA indicated a significant effect of factor 1 (Funtional group) for some leaf traits ($p < 0.05$) and a non-significant effect of factor 2 (remnants of Atlantic Forest) for all leaf traits ($p > 0.05$). Non-significant interactions were observed between factors 1 and 2 ($p > 0.05$). Distinct letters indicate significant differences between both functional groups ($p < 0.05$), according to the pairwise multiple comparison method applied (Holm-Sidak test).

| **Leaf traits** | **Pioneer species** | **Non-pioneer species** | **p-Values (Two way ANOVA)** | | |
|---|---|---|---|---|---|
| **Morphological traits** | | | Factor 1 | Factor 2 | Factor 1x2 |
| LA ($cm^2$)* | 56.31 (±42.44) | 100. 14 (±240.26) | 0.321 | 0.093 | 0.650 |
| LMA (mg $cm^{-2}$) | 15.90 (±9.75)a | 8.98 (±4.8)b | 0.038 | 0.659 | 0.234 |
| LD (mg $cm^3$) | 0.66 (±0.23)a | 0.41 (±0.12)b | 0.004 | 0.347 | 0.921 |
| DMC (mg $cm^{-3}$) | 2.52 (±1.76)a | 1.80 (±1.25)a | 0.2 | 0.416 | 0.092 |
| RWC (%) | 74.79 (±12.44)a | 69.61 (±14.44)a | 0.355 | 0.366 | 0.645 |
| **Anatomical traits** | | | | | |
| LT (μm) | 196.86 (±84.09)a | 210.81 (±82.42)a | 0.689 | 0.219 | 0.226 |
| PP (μm) | 86.08 (±30.13)a | 58.06 (±22.93)b | 0.001 | 0.003 | 0.187 |
| SP (μm) | 73.65 (±58.78)b | 117.28 (±67.99)a | 0.041 | 0.405 | 0.433 |
| IS (%) | 14.57 (±9.49)a | 21.02 (±8.15)a | 0.067 | 0.091 | 0.918 |
| Abaxial surface SD ($mm^2$) | 15.90 (±9.75)a | 8.98 (±4.8)b | 0.042 | 0.58 | 0.586 |
| Abaxial surface TD ($mm^2$) | 16.77 (±17.48)a | 5.08 (±5.01)b | 0.051 | 0.928 | 0.602 |

* Comparison performed by the non-parametric Mann-Whitney test due to the absence of normal

**Table 4.** Morphology and distribution of leaf epicuticular waxes and trichomes of pioneer species (P) and non-pioneer species (NP) sampled at the Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI). (-) indicates the difficulty to observe the cuticle due to the dense distribution of trichomes.

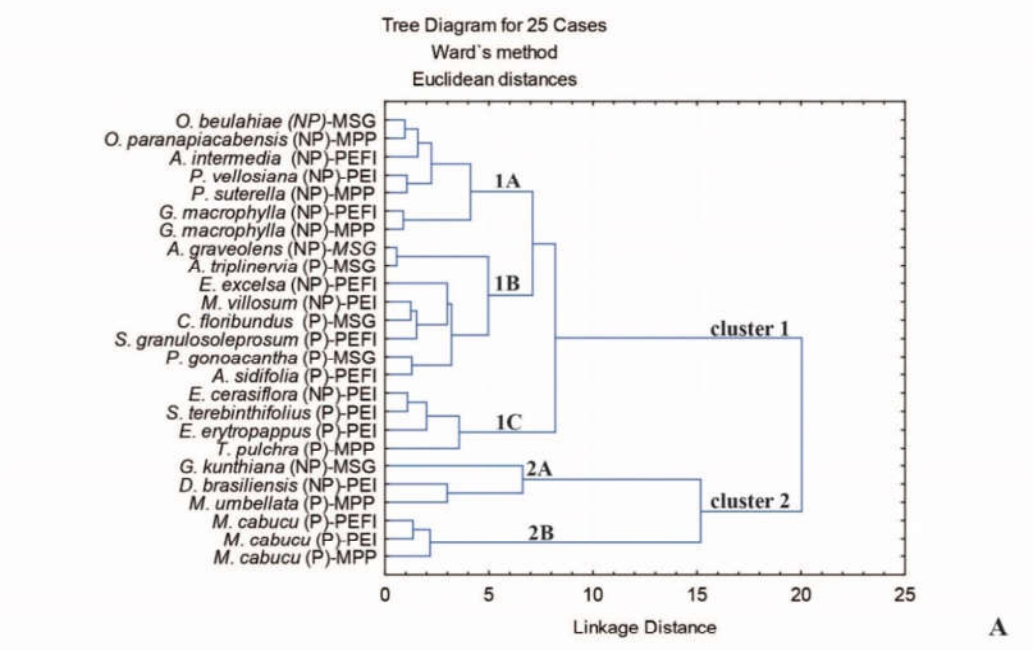
| Family/ Species (funtional group) | Site | Leaf epicuticular waxes | | Trichomes | | Figures |
|---|---|---|---|---|---|---|
| | | Adaxial surface | Abaxial surface | Adaxial surface | Abaxial surface | |
| Anacardiaceae | | | | | | |
| *Astronium graveolens* Jarcq.(NP) | MSG | striated | smooth and striated close to stomata and trichomes | non-glandular, branched | non-glandular, branched | S1L and X |
| *Schinus terebinthifolia* Raddi (P) | PEI | striated | striated | glabrous | glandular | S1K and Y |
| Asteraceae | | | | | | |
| *Eremanthus erytropappus* (DC.) MecLeisch (P) | PEI | smooth | platelets with projections at acute angle | non-glandular, unbranched, and glandular | glabrous | 1D and M |
| Euphorbiaceae | | | | | | |
| *Alchornea sidifolia* Müll. Arg. (P) | PEFI | smooth | smooth and striated close to stomata and trichomes | non-glandular, branched | non-glandular, branched | 1B and K |
| *Alchornea triplinervia* Müll. Arg. (P) | MSG | striated | striated | non-glandular, branched | non-glandular, branched | 1H and Q |
| *Croton floribundus* Spreng. (P) | MSG | smooth | – | non-glandular, unbrached and branched | non-glandular, branched | S1G and T |

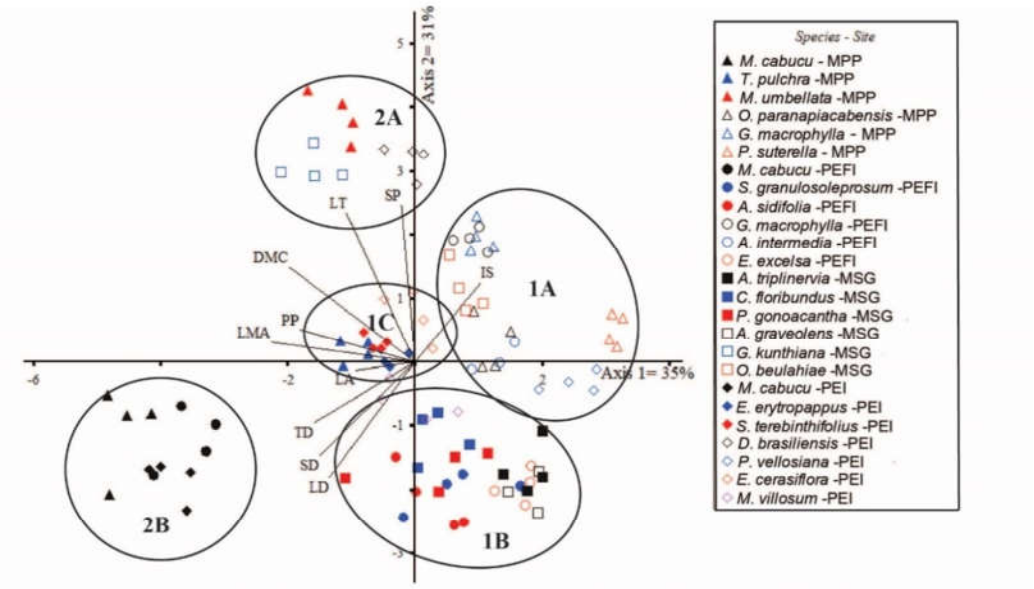
| | | | | | | |
|---|---|---|---|---|---|---|
| Fabaceae | | | | | | |
| *Machaerium villosum* Vogel (NP) | PEI | smooth | platelets | non-glandular, unbranched | non-glandular, unbranched | S1F and S |
| *Piptadenia gonoacantha* (Mart.) J.F.Macbr.(P) | MSG | platelets | platelets | non-glandular, unbranched | non-glandular, unbranched | 1I and R |
| Lauraceae | | | | | | |
| *Ocotea beulahiae* J.B. Baitello (NP) | MSG | smooth | smooth | glabrous | glandular | 1A and J |
| *Ocotea paranapiacabensis* Coe-Teixeira (NP) | MPP | smooth | smooth | glabrous | non-glandular, unbranched | S1D and Q |
| Melastomataceae | | | | | | |
| *Miconia cabucu* Hoehne (P) | MPP, PEFI and PEI | smooth | – | glabrous | non-glandular, branched | S1C and P |
| *Tibouchina pulchra* Cong (P) | MPP | smooth | smooth and striated close to stomata | non-glandular, unbranched | non-glandular, unbranched and glandular | 1E and N |
| Meliaceae | | | | | | |
| *Guarea kunthiana* A. Juss. (NP) | MSG | striated | smooth | glabrous | non-glandular, unbranched | S1J and W |
| *Guarea macrophylla* Vahl (NP) | MPPand PEFI | smooth and striated close to trichome | smooth combined with wax deposits forming cuticle | non-glandular, unbranched | non-glandular, unbranched and glandular | 1G and P |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | elevations | | | |
| *Myrsinaceae* | | | | | | |
| *Myrsine umbellata* Mart. (P) | MPP | smooth | striated | glandular | glandular | S1I and V |
| Myrtaceae | | | | | | |
| Amaioua intermedia *Mart. ex Schult. & Schult.f* (NP) | PEFI | smooth | smooth | glabous | non-glandular, unbranched | S1A and N |
| *Eugenia cerasiflora* Miq. (NP) | PEI | smooth | smooth | glandular | glandular | S1H and U |
| *Eugenia excelsa* O. Berg (NP) | PEFI | smooth | smooth | glabrous | glabrous | S1B and O |
| Rubiaceae | | | | | | |
| *Psychotria suterella* Müll. Arg.(NP) | MPP | striated | smooth | glandular | non-glandular, unbranched trichomes | S1M and Z |
| *Psychotria vellosiana* Benth (NP) | PEI | smooth | smooth | glabrous | non-glandular, unbranched | S1V and R |
| Solanaceae | | | | | | |
| *Solanum granulosoleprosum* Dunal (P) | PEFI | smooth | – | non-glandular, unbranched and branched | non-glandular, branched | 1F and O |
| Winteraceae | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Drimys brasiliensis Miers (NP) | PEI | smooth | platelets with projections on rounded granules | glandular | glabrous | 1C and L |

**Fig. 1.** Multivariate analyses performed with the morphological and anatomical leaf trait of 12 pioneer (P) and 13 non-pioneer (NP) tree species sampled at the Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI). A: tree dendrogram resulting from cluster analysis. B: graphical representation of the principal component analysis (PCA). LA, leaf area; LMA, leaf mass area; LD, leaf density; DMC, dry matter concentration; LT, leaf thickness; PP, palisade parenchyma thickness; SP, spongy parenchyma thickness; IS, intercellular space; SD, abaxial stomatal density; TD, abaxial trichomes density. Filled symbols refer to pioneer species; Unfilled symbols refer to non-pioneer species. The clusters 1 and 2 originated from cluster analysis are highlighted in the figure (B). The table shows the correlation coefficients of each variable to axis 1 and 2.

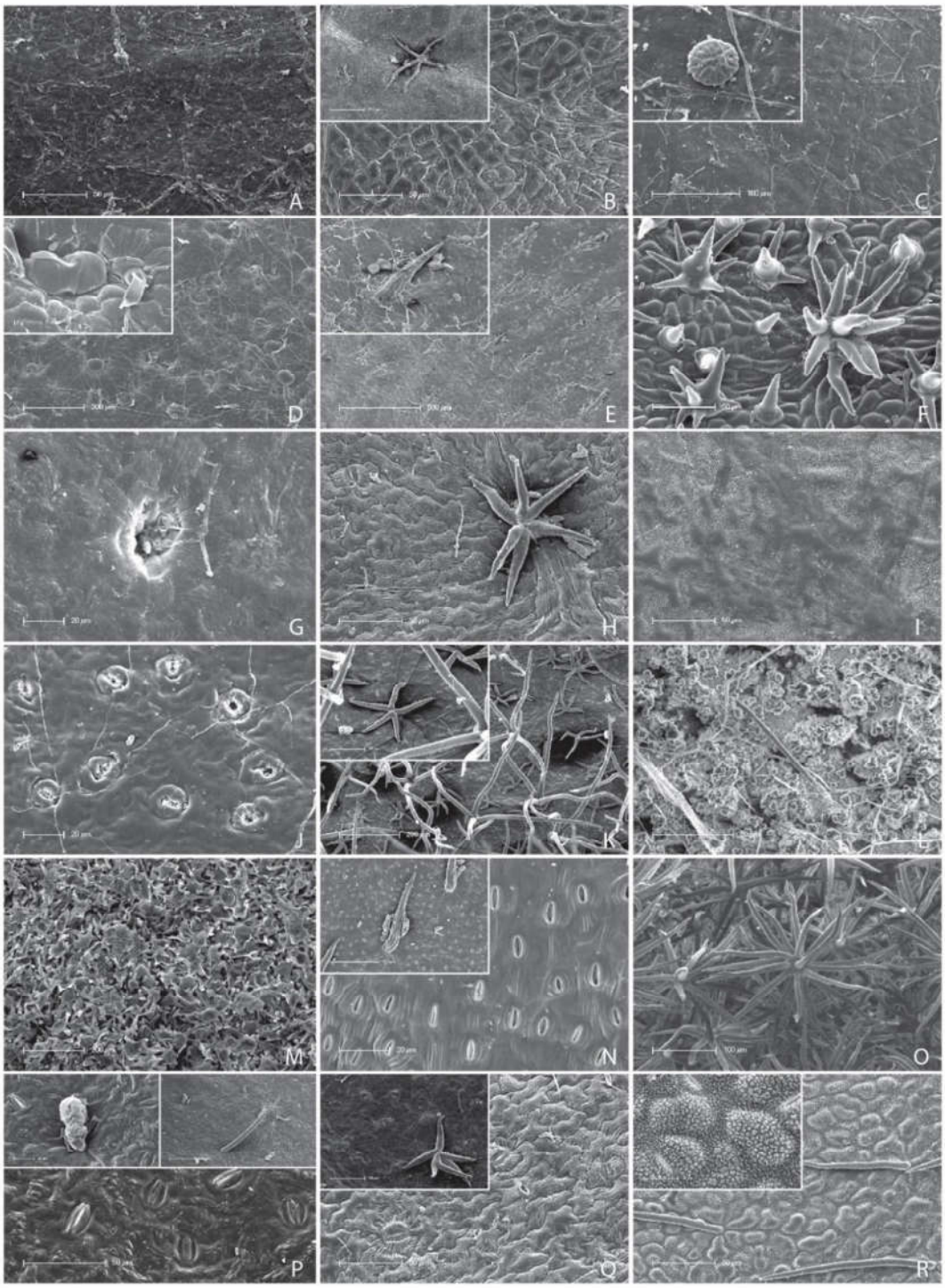



| | LA | LMA | LD | DMC | LT | PP | SP | IS | SD | TD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Axis 1** | -0.37 | -0.91 | 0.54 | -0.79 | -0.43 | -0.72 | -0.08 | 0.41 | 0.60 | -0.63 |
| **Axis 2** | -0.05 | 0.11 | -0.67 | 0.55 | 0.88 | 0.19 | 0.92 | 0.47 | -0.55 | 0.36 |

B

**Fig. 2.** SEM of the leaf surface of some tree species from different remnants of the Atlantic Forest. A-J: Adaxial surface. K-R: Abaxial surface. A. Smooth cuticle and glabrous surface (*Ocotea beulahiae*). B (*inset*). stellar non-glandular trichomes (*Alchornea sidifolia*). C (*inset*). peltate glandular trichomes (*Drimys brasiliensis*). D (*inset*). simple non-glandular trichomes covered by a smooth cuticle (*Eremanthus erytropappus*). E (*inset*). simple non-glandular trichomes (*Tibouchina pulchra*). F. Stellar and simple non-glandular trichomes covered by a smooth cuticle (*Solanum granulosoleprosum*). G. Smooth and striated cuticle next to trichome (*Guarea macrophylla*). H. Stellar non-glandular trichomes covered by a striated cuticle (*Alchornea triplinervia*). I. Wax deposition in platelets (*Piptadenia gonoacantha*). J. A glabrous surface covered by a smooth cuticle (*Ocotea beulahiae*). K and N. Smooth and striated cuticle over stomata and non-glandular trichomes. Note the simple trichomes (K), and stellar trichomes (N). K (*inset*) *Alchornea sidifolia;* N (*inset*) *(Tibouchina pulchra*). L. Deposition of waxes in platelets and projections in rounded granules *(Drimys brasiliensis*). M. Deposition of waxes in platelets and projections in acute angles (*Eremanthus erytropappus*). O. Stellar non-glandular trichomes densely distributed at different heights over the leaf surface (*Solanum granulosoleprosum*). P (*inset*). Deposition of waxes forming elevations in the cuticle, and hairy surface composed by glandular and simple non-glandular trichomes (*Guarea macrophylla*). Q (*inset*). Stellar non-glandular trichomes and striated cuticle (*Alchornea triplinervia*). R (*inset*). Deposition of waxes in platelets, and very long non-glandular trichomes dispersed over the leaf blade (*Piptadenia gonoacantha*). In A, C, D, F, H and J. Note the particles adhered to fungal hyphae (A. C, D, F, H) and particles obstructing the stomatal pore (J).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## 4. Discussion

The subtropical tree species included in this investigation were mostly grouped according to their functional group. A cluster was formed by non-pioneer species (cluster 1A), and other exclusively by one pioneer species (cluster 2B). The last three groups were encompassed by a mixture of pioneer and non-pioneer species (cluster 1B and 1C with a predominance of pioneer trees, and cluster 2A with a predominance of non-pioneer trees). The species in each group were associated by combinations of morpho-anatomical leaf traits indicative of distinct potential tolerance levels to natural or anthropic environmental stressors. We ordinated the groups from the most to the least potential tolerant to environmental stressors according to the following sequence: cluster 2B > cluster 1C > cluster 1B > cluster 2A = cluster 1A, as will be discussed below.

We categorized *M. cabucu* (cluster 2B), followed by *E. erytropappus*, *S. terebinthifolius* and *T. pulchra* (cluster 1C) as the most potentially tolerant species to oxidative stress based on their high leaf mass per area (LMA), dry matter concentration (DMC), palisade parenchyma (PP) among other morphological/anatomical functional traits. These leaf traits basically avoid the action of the environmental stressors. *M. cabucu* and *T. puclhra*, both belonged to Melastomataceae family, also have physiological and biochemical leaf traits that restrict the effects of oxidative stress (Brandão et al., 2017), revealing a high phenotypic plasticity that allows them to survive under a wide variety of environmental conditions (Bussoti and Polastrini, 2015).

The LMA is the most responsive leaf trait to distinguish between sensitive (low LMA) and tolerant species (high LMA) to oxidative stress imposed by gaseous pollutants, more specifically ozone (Bussotti et al., 2008; Calatayud et al., 2011; Li et

al., 2016) or drought and high solar radiation (Poorter et al., 2009; Bussotti and Pollastrini, 2015; de la Riva et al., 2016). A high LMA results from thick leaves (indicated by LT values) and/or a dense mesophyll (LD or DMC values) (Bussotti, 2008), and also from anatomical traits (de la Riva et al., 2016), such as large cells, high number of mesophyll layers, low fraction of intercellular spaces (John et al., 2017), and thick cell walls (Bussotti et al., 1995; Bussotti, 2008; Bussotti and Pollastrini, 2015). The high values of DMC may be related to an increase of the total structural carbohydrates and lignin (Poorter et al., 2009). High values of DMC in these tolerant species seemed to be a consequence of the high proportion of PP, which compacts the mesophyll and reduces the intercellular spaces (Bussotti, 2005; Niinemets, 1999), increasing the resistance to the diffusion of atmospheric pollutant gases, as observed Bennett et al. **(**1992) in ozone-tolerant species.

The leaf surface is the first contact with excessive solar radiation and other climatic stressors and with atmospheric pollutants. The high density of stomata in the tolerant species evaluated here (*M. cabucu,* the most tolerant, cluster 2B*,* and within the cluster 1C) can provide the increase of stomatal conductance and absorption of air pollutants under favorable environmental conditions, as discussed by Ferdinand et al., (2000). However, other studies have reported that high stomatal density may lead to tolerance to gaseous pollutants, assuming that the increase in stomatal density leads to a lower conductance per single stoma (Pääkkönen et al., 1997), neutralizing the deleterious gaseous pollutants effect (Paoletti and Grulke, 2005). The same species that were correlated with high stomatal density also presented high density of trichomes in the abaxial leaf blade surface (again, mainly in the most tolerant *M. cabucu*), which act as a physical barrier against the entrance of gaseous pollutants (Roschina and Roschina, 2003; Cardoso-Gustavson et al., *in prep.*), and may be an

efficient mechanism to avoid oxidative stress. Specially, the glandular trichomes observed in the abaxial leaf surface of tree species included in the cluster 1C (*E. ceraciflora*, *S. terebenthifolia*, *T. pulchra*) may act as an antioxidant barrier, storing and releasing metabolites such as flavonoids and VOC (Agati et al., 2012; Li et al., 2018). Indeed, as pointed by Jud et al. (2016) and Li et al. (2018), glandular trichomes improve oxidative resistance, contributing to the reduction of ozone toxicity.

The retention of particulate matter (PM) depends on the characteristics of the leaf surface (Saebo et al., 2012; Mo et al., 2015; Sánchez-Lopez et al., 2015). The toxic elements and compounds adsorbed to PM (e.g. metals, nitrate, iron, sulfates, polycyclic aromatic hydrocarbons) can be adsorbed to stomata, trichomes and epicuticular waxes and further penetrate inner tissues (Saebo et al. 2012; Simon et al. 2014; Sánchez-Lopez et al. 2015). Considering the roughness of the leaf surface caused by the high trichome density and epicuticular waxes ornamentation, the tree species included in cluster 2B (*M. cabucu*) and cluster1C (*E. erytropappus*, *S. terebinthifolius* and *T. pulchra*) have the highest potential for PM accumulation. *M. cabucu* (cluster 2B) seems also to be a good particle accumulator due to the high abundance of non-glandular trichomes with a wide area at the abaxial surface, in spite of the glabrous adaxial surface. However, the species that showed the greatest potential of PM accumulation on leaf surface are not necessarily the most susceptible to the stress induced by toxic components adsorbed on the particles that entered the leaves by passive or active processes. The level of the PM-derived oxidative stress also depends on the mechanisms of resistance to the entrance of these pollutants into the foliar tissues (previously discussed) and detoxification (Domingos et al., 2015). A similar interpretation about the species tolerance to PM can be extrapolated to

oxidative stress caused by gaseous pollutants. *M. cabucu* was considered the most tolerant species to oxidative stress caused by aerosol, because it has the highest cuticle thickness (data not show), tissue thickness and compact mesophyll comparing to the other species evaluated here in. *E. erytropappus*, *S. terebinthifolius* and *T. pulchra* (cluster 1C) may be recognized as intermediate tolerant to aerosols because they have a compact structure, besides the greater capacity to accumulate PM.

In contrast, the species included in the cluster 1A, all of them classified as non-pioneers (*A. intermedia, G. macrophylla, O. beulahiae, O. paranapiacabensis, P. suterella* and *P. vellosiana*), presented opposite characteristics of leaf structure (low LMA, DMC and PP) and leaf surface (low stomatal density of the abaxial surface, SD and trichome density of the abaxial surface, TD) relative to those from clusters 2B e 1C and was recognized as potentially more vulnerable to oxidative stress. In addition, *A. intermedia, G. macrophylla, O. beulahiae, O. paranapiacabensis, P. suterella* and *P. vellosiana* (cluster 1A), which have leaves with smooth cuticle, glabrous surface and/or low trichomes density, seemed to be less efficient in particle capture and/or sensitive to the PM-derived oxidative stress due to their intrinsic anatomical features. A high particle retention on the stomata pores of *O. beulahiae* and *O. paranapiacabensis* may be related to the morphology and chemical composition of the waxes deposited in the cuticle of the guard cells. The obliteration of the stomata by PM in these species can directly impact photosynthesis due to changes in stomatal conductance, increase of leaf temperature, and decrease in transpiration rate (Pereira et al., 2009). Moreover, the non-pioneer species included in the cluster 1A (*O. beualahiae* and*, G. macrophylla*), also have low antioxidative capacity (low ascorbic acid ratio and/or glutathione ratio) and a high oxidative damage (high levels of hydroperoxide conjugated diene) (Brandão et

al., 2017), reinforcing their potential sensitivity to oxidative stress.

The morpho-anatomical characteristics of species included in the cluster 1B (*A. sidifolia, A. triplinervia*, *C. floribundus, P. gonoacantha, S. granulosoleprosum*, *A. graveolens, E. excelsa* and *M. villosum*) indicated an intermediate potential of tolerance to environmental stressors. These species showed a high LD, and a low LT and SP. In addition, non-glandular trichomes observed in all intermediate species occupy a large surface area (Li et al., 2018), and contribute to reduce the interception of solar radiation and confer UV protection (Bickford, 2016), and strongly modulate boundary layer resistance (Bickford, 2016), increasing the chemical leaf resistance by confining and increasing the concentration the volatile organic compounds (VOC) in the boundary layer, avoiding the entrance of the gaseous pollutant into the leaf (see in *C. floribundus* - Cardoso-Gustavson et al., 2014). The ornamentation of the epicuticular waxes (striated or platelets) also facilitated the PM accumulation on the leaf surface of some tree species intermediate potential of tolerant to oxidative stress studied. The morphology and chemical composition of the epicuticular waxes are important features to define the bioaccumulating properties of some species (Saebo et al., 2012; Dias et al., 2015; Domingos et al., 2015).*M. umbellata*, *D. brasiliensis* and *G. kunthiana* (cluster 2A) showed opposite characteristics (low LD, high LT and SP), indicative of a higher potential sensitivity to oxidative stress compared to the species of cluster 1B. A higher SP thickness facilitates the diffusion of gases inside the leaf (dos Anjos et al., 2015; Tian et al., 2016) and PM-derived oxidative stress. Although a high LD is related to a dense mesophyll, with few intercellular spaces (similar to tolerant species), an opposite response is expected when a high LD is combined with a thick SP (Bussotti, 2005). The higher LT observed in *M. umbellata*, *D. brasiliensis* and *G. kunthiana* associated with a higher SP thickness, resulted in

low LD leaves that impose less resistance to the diffusion of gaseous pollutants (Ferdinand, 2000). In this case, the lower the LD, the higher is the possibility of gaseous pollutants affect negatively the PP (Bennett et al., 1992; Pääkkömen et al., 1997).

*M. umbellata* and *D. brasiliensis* showed a greater capacity of water absorption by the leaf blade – a feature that favors the performance of plants in seasonally dry environments (Eller et al., 2013, 2016). They are susceptible to the turgor loss of leaf cells during the dry seasons due to their anisohydric characteristics (Eller et al., 2016). Since both species keep their stomata open even in adverse conditions, they would be more vulnerable to climate change (Eller et al., 2016) and the entrance of gaseous pollutants.

In brief, the pioneer species investigated (all included in the clusters 2B, 1C or 1B) produce leaves with high LMA, LD, PP, SD (abaxial surface) and TD (abaxial surface) that confer intermediate to high potential tolerance against environmental stressors in the subtropics, such excessive solar radiation and air pollutants. These characteristics contrast with the morpho-anatomical traits described for non-pioneer trees (mostly included in the clusters 2A and 1A) that evidenced a low potential tolerance. These results confirm our first hypothesis and are in accordance with the definition presented by Favaretto et al. (2011). They affirm that the native tree species in tropical/subtropical forests can be classified into two major ecological groups, based on the requirement of light and acclimatization to shading: pioneer species (shade intolerant) and non-pioneer species (shade tolerant).

Our results here contrast with those described by Bussotti et al. (2008), who concluded that pioneer species under Mediterranean climate have low degree of acclimatization and low efficiency in the process of detoxification due to low LMA,

low LD, low ability to produce defense compounds, few anatomical mechanisms, low water use efficiency, high photosynthetic rate and low leaf longevity. Bussotti (2008) also commented that the leaf traits of both functional groups vary in relation to low rainfall and fertility. However, Mediterranean forests are exposed to different natural environmental stressors in relation to tropical forests, which seem to contribute to explain these contradictory results. The high solar irradiance throughout the year in in the tropical region studied seems to have a stronger influence over the physiological performance of the species (Favaretto et al., 2011; Aguiar-Silva et al., 2016; Esposito et al., 2018) than the water availability to the plants, as the leaf water status did not varied significantly among species.

The tree species investigated were not ordinated according to the forest of origin by the cluster analysis, indicating that their morpho-anatomical leaf traits did not change in function to the climatic gradient observed among the MPP region, where a dry season does not exist, and MSG and PEI regions, where a marked dry season is evidenced. The distinct sources and levels of air pollutants seemed also not influence the grouping of species. Therefore, our second hypothesis was not confirmed based on the morpho-anatomical traits measured. This affirmation is reinforced by the fact that trees of *M. cabucu* sampled in three forest remnants (MPP, PEFI, PEI) and of *G. macrophylla* sampled in two forests remnants (MPP, PEFI) were included in the same clusters (2B and 1A respectively).

The absence of spatial variation may suggest the influence of the evolutionary history of the evaluated species. This assumption seems to be reinforced by two other aspects: (1) *E. ceraciflora*, *M. vilossum*, *E. excelsa* and *A. graveolens* (included in the clusters 1B and 1C) have been classified as non-pioneer trees in many ecological studies (e.g. Guaratini et al., 2008; Lima et al., 2011), but have morpho-anatomical

characteristics similar to those of pioneer species; (2) *M. umbellata* classified as pioneer species (Lima et al., 2011) was included in the cluster 2A together with other two non-pioneer species.

Therefore, we also focused our discussion on the phylogenetic aspects of target-taxons to evaluate the species adaptation to the environmental stresses, following the recent tendency of developing phylogenetic tools to bioassessment and ecotoxicology (Keck et al., 2016). This new focus may aid further studies aiming at searching for indicator taxa of atmospheric pollutant in tropical forests. We based on the fact that environmental filters (abiotic constraints) restrict the presence of species within a community of individuals that bear specific phenological, morphological and physiological trait values (Gestauer et al., 2017). Close-related species would share similar functional traits, although some trait values may be homoplastic, *i.e*., the same pattern may exist in phylogenetically distant species. We are also aware that that our samples are limited considering the amplitude of some families (for example, Fabaceae). Anyway, our study is the first attempt to find a model of responses of Atlantic forest trees to anthropogenic changes.

The ecological data obtained herein was compared with an optimized angiosperm supertree (Fig. 3), looking for indications of phylogenetic signals above the specific level (family). A phylogenetic distinction was observed among the families whose representatives included in this study are potentially tolerant (Euphorbiaceae, Fabaceae, Myrtaceae, Melastomataceae, Solanaceae, Asteraceae) or vulnerable (Winteraceae, Lauraceae, Myrsinaceae, Rubiaceae) to environmental constrains. The only exception observed here was Anacardiaceae (Sapindales), in which both tolerant and vulnerable representatives were identified in the different remnants.

The ecological data obtained here support the vulnerability potential of shade-tolerant Magoliids, here represented by Winteraceae (*Drymis*) and Lauraceae (*Ocotea*). Indeed, the basal order Magnoliales and Winteraceae were interpreted in the past as ecological models of basal angiosperms due to their large leaves, slow-growing trees, and constitute the understorey of tropical forests (Carlquist, 1975). In addition, our data indicate that representatives of Magnoliids are vulnerable to pollutants and other environmental stresses. However, some studies have been suggested that basal angiosperms were able to survive in both shaded and sunny habitats according to their lineage (Lee et al., 2015 and references therein).

However, *O. beualahiae* and *O. paranapiacabensis* are endemic species in the Atlantic forest portions that occur in SE Brazil [São Paulo and Espirito Santo (only *O. beualahiae*) States (Flora do Brasil 2020, under construction)]. A lower plasticity of responses to different environmental conditions is expected in relation to widely distributed species due to the small geographical distribution of these species. *O. paranapiacabensis* occurs in a low disturbed environment (reference forest - MPP), therefore, naturally less acclimated to stress conditions. Indeed, they are more vulnerable to environmental changes and have a more restrict distribution comparing to Melastomataceae and other species herein defined as tolerant.

In contrast, events of convergent and divergent evolution in the past were responsible for the evolution of the morphological and anatomical leaf traits related to water use efficiency, water conservation and conservation of resources, allowing the adaptation and wide distribution (including hostile environments) of Melastomataceae species (Castro, 2015). Besides, the high reproductive success in *Miconia* due to self-pollination and production of a large number of seeds facilitate the rapid propagation of its species and invasion of large areas (Medeiros et al.,

1997; Hardesty et al., 2009); generating descendants even in restricted environments.

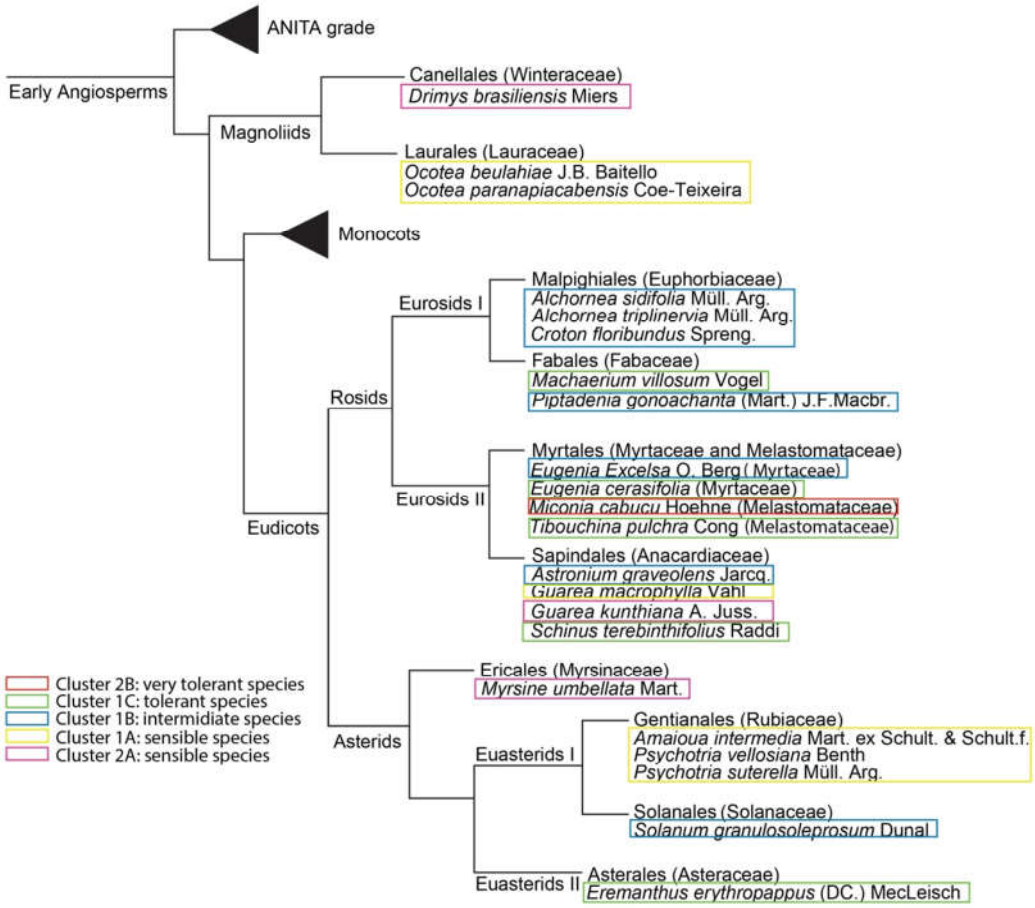


**Fig. 3.** Simplified supertree of Angiosperm phylogeny based on APG III (2009) and APG IV (2016), showing orders, families and species included in the present study. The species were grouped according to the results of cluster analyses presented in Fig. 2 and classified according to their potential vulnerability to environmental stressors (very tolerant to sensitive species).

## 1. Conclusions

The morphological and anatomical leaf traits were useful for grouping species according to their tolerance potential. Pioneer tree species also have morpho-anatomical traits that restrict or avoid the effects of oxidative stress posed by natural

and anthropic stressors than non-pioneer species. We confirmed this hypothesis based on the fact that pioneer trees species presented leaves with high leaf mass area (LMA), leaf density (LD), palisade parenchyma thickness (PP), abaxial stomatal density (SD) and abaxial trichomes density (TD). The pioneer species have high conditions of resilience and of perpetuating in the disturbed Atlantic Forest remnants, mainly Melastomataceae species. Non-pioneer trees species that evidenced a low potential tolerance with a higher spongy parenchyma thickness (SP). In general, non-pioneer species are less adapted and less acclimated to the oxidative stress imposed by environmental conditions and have high chances of a rapid extinction in the disturbed Atlantic forest.

The second hypothesis that the morpho-anatomical traits are more evident in tree species from forest remnants exposed to more extreme environmental conditions was rejected. The absence of a spatial variation of the morpho-anatomical traits analyzed here in a climatic and altitudinal gradient at remnants of the Atlantic Forest may result from the characteristics coming from the evolutionary history of the species, which would provide greater or lesser plasticity for occupation of different environments or as responses to environmental disturbances.

Evidences of a phylogenetic signal above the specific level (family) were detected. Our study offers new insights, but further confirmation is required since our sample is very limited when considering the size of families.

**Acknowledgements**

The authors gratefully the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing a PhD scholarship to the first author; Municipal Park Paranapiacaba, State Park Fontes do Ipiranga, Ecological Area of

Relevant Interest Mata de Santa Genebra and State Park Itacolomi for permitting the leaf collections; Dr. Eduardo Pereira Cabral Gomes, Dr. Hildeberto Caldas de Sousa and Dr. Maria Cristina Teixeira Braga for helping in tree species selection; Amariles C. de Souza, Douglas D. Santos, Giovanna Boccuzzi, Marisia P. Esposito, Marcela R.G.S. Engela, Ricardo K. Nakazato and Tiago A. Tassinari for the assistance during field work.

## 6. References

Agati, G., Azzarrello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci 196, 67–76.

Aguiar-Silva, C., Brandão, S.E., Bulbovas, P., 2016. Antioxidant responses of Atlantic Forest native tree species as indicators of increasing tolerance to oxidative stress when they are exposed to air pollutants and seasonal tropical climate. Ecol. Indic. 63, 154–164.

APG - Angiosperm Phylogeny Group III. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Bot. J. Linn. Soc. 161, 105–121.

APG - Angiosperm Phylogeny Group IV. 2016. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Bot. J. Linn. Soc. 181, 1–20.

Barrs, H.D., Weatherley, P.E., 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust J Biol Sci 15, 413–428.

Bennett, J.P., Rassat, P., Berrang, P., Karnosky, D.F., 1992. Relationships between leaf anatomy, and ozone sensitivity of *Fraxinus pennsylvanica* marsh. and *Prunus serotina* Ehrh. Environ. Exp. Bot. 32, 33–41.

Bickford, C., 2016. Ecophysiology of leaf trichomes. Funct Plant Biol. 43, 807–814.

Brandão, S.E., Bulbovas, P., Lima, M.E., Domingos, M., 2017. Biochemical leaf traits as indicators of tolerance potencial in tree species from the Brazilian Atlantic Forest against oxidative environmental stressors. Sci. Total Environ. 575, 406–417.

Bussotti, F., 2005. Leaf morphology and chemistry in *Fagus sylvatica* (beech) trees as affected by site factors and ozone: results from CONECOFOR permanent monitoring plots in Italy. Tree Physiol. 25, 211–219.

Bussotti, F., 2008. Functional leaf traits, plant communities and acclimation processes in relation to oxidative stress in trees: a critical overview. Glob. Chang. Biol. 14, 2727–2739.

Bussotti, F., Bottacci, A., Bartolesi, A., Grossoni, P., Tani, C., 1995. Morpho-anatomical alteration in leaves collected from beech trees (*Fagus sylvatica* L.)

in conditions of natural water stress. Environ. Exp. Bot. 35, 201–213.

Bussotti, F., Pollastrini, M., 2015. Evaluation of leaf features in forest trees: Methods, techniques, obtainable information and limits. Ecol Indic. 52, 219–230.

Calatayud, V., García-Breijo, F.J., Cervero, J., Reig-Armiñana, J., Sanz, M.J. 2011., Physiological, anatomical and biomass partitioning responses to ozone in the Mediterranean endemic plant Lamottea dianae. Ecotoxicol. Environ. Saf. 74, 1131–1138.

Cardoso-Gustavson, P., Bolsoni, V.P., de Oliveira, D.P., Guaratini, M.T., Aidar, M.P., Marabesi, M.A., Alves, E.S., de Souza, S.R., 2014. Ozone-induced responses in *Croton floribundus* Spreng. (Euphorbiaceae): metabolic cross-talk between volatile organic compounds and calcium oxalate crystal formation. PloS One 9 (8), e105072.

Cardoso-Gustavson, P., Moura, B.B., Pedrosa, G.S., Centeno, D.C., Souza, S.R., The role of leaf hairs in avoiding ozone uptake**.** Environ Sci Pollut Res (*in press*).

Carlquist, S. 1975. Ecological strategies of xylem evolution. University of California Press, Berkeley.

Castro, S.A.B., 2015. Ecofisiologia foliar de Melastomataceae dos campos rupestres. (PhD thesis). Universidade Federal de Minas Gerais, Belo Horizonte-MG.

de la Riva, E.G., Olmo, M., Poorter, H., Ubera, J.L., Villar, R., 2016. Leaf mass per area (LMA) and its relation with leaf structure and anatomy in 34 Mediterranean woody species along water availability gradient. PLoS One 11 (2), e0148788.

Dias, A.P.L., Rinaldi, M.C.S., Domingos, M., 2016. Foliar accumulation of polycyclic aromatic hydrocarbons in native tree species from the Atlantic Forest (SE-Brazil). Sci Total Environ. 544, 175–184.

Díaz, S., Purvis, A., Cornelissen, J.H.C., Mace, G.M., Donoghue, M.J., Ewers, R.M., Jordano, P., Pearse, W.D., 2013. Functional traits, the phylogeny of function, and ecosystem service vulnerability. Ecol. Evol. 3, 2958–2975.

Domingos, M., Bulbovas, P., Camargo, C.Z.S., Aguiar-Silva, C., Brandão, S.E., Dafré-Martinelli, M., Dias, A.P.L., Engela, M.R.G.S., Gagliano, J., Moura, B.B., Alves, E.S., Rinaldi, M.C.S., Gomes, E.P.C., Furlan, C.M., Figueiredo, A.M.G., 2015. Searching for native tree species and respective potential biomarkers for future assessment of pollution effects on the highly diverse Atlantic Forest in SE-Brazil. Environ. Pollut. 202, 85–95.

Domingos, M., Klumpp, A., Klumpp, G., 2003. Disturbances to the Atlantic Rain Forest in Southeast Brazil. In: Emberson, L., Ashmore, M., Murray, F. (Eds.), Air Pollution Impacts on Vegetation. A Global Assessment. Air Pollution Reviews vol. 4. Imperial College Press, London, pp. 287-3–8.

dos Anjos, L., Oliva, M.A., Kuki, K.N., Mielke, M.S., Ventrella, M.C., Galvão, M.F., Pinto, L.R.M., 2015. Key leaf traits indicative of photosynthetic plasticity in tropical tree species. Trees 29, 247–258.

Eller, C.B., Lima, A.L., Oliveira. R.S. 2013. Foliar uptake of fog water and transport belowground alleviates drought effects in the cloud forest tree species, *Drimys brasiliensis* (Winteraceae). New Phytol 199, 151–162.

Eller., C.B., Lima, A.L., Oliveira, R.S., 2016. Cloud forest trees with higher foliar water uptake capacity and anisohydric behavior are more vulnerable to drought and climate change. New Phytol 211, 489–501.

Esposito MP, Pedroso ANV, Domingos M., 2016. Assessing redox potential of a native tree from the Brazilian Atlantic Rainforest: a successful evaluation of oxidative stress associated to a new power generation source of an oil refinery. Science of the Total Environment 550, 861–870.

Esposito, M.P., Nakazatu., R.K., Pedroso, A.V.P., Lima, M.E.L., Figueiredo, M.A., Diniz, A.P., Kozovits, A.R., Domingos, M., 2018. Oxidant-antioxidant balance and tolerance against oxidative stress in pioneer and non-pioneer tree species from the remaining Atlantic Forest. Sci Total Environ. 625, 382–393.

Esquivel-Muelbert, A., Galbraith, D., Dexter, K.G., Baker, T.R., Lewis, S.L., Meir, P., Rowland, L., Costa, A.C.L., Nepstad, D., Phillips, O.L., 2017. Biogeographic distributions of neotropical trees reflect their directly measured drought tolerances. Sci Rep. 21; 7 (1), 8334.

Favaretto, V.F., Martinez, C.A., Soriani, H.H., Furriel, R.P.M., 2011. Differential responses of antioxidant enzymes in pioneer and late-successional tropical tree species grown under sun and shade conditions. Environ. Exp. Bot. 70, 20–28.

Feder, N., O'Brien., 1968. Plant Microtechnique: Some Principles and New Methods. Am J Bot. 55, 123–142.

Ferdinand, J.A., Fredericksen, T.S., Kouterick, K.B., Skelly, J.M., 2000. Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. Environ. Pollut. 108, 297–302.

Flora do Brasil. 2020. under construction. Jardim Botânico do Rio de Janeiro. Available at: <http://floradobrasil.jbrj.gov.br/>.

Gestauer, M., Saporetti-Junior, A.W., Valladares, F., Meira-Neto, J.A.A., 2017. Phylogenetic community structure reveals differences in plant community assembly of an oligotrophic white-sand ecosystem from the Brazilian Atlantic Forest. Acta Bot. Bras. 31, 531–538.

Guaratini, T.M.G., Gomes, E.P.C., Tamashiro, J.T., Rodrigues, R.R., 2008. Composição florística da Reserva Municipal de Santa Genebra. Braz. J. Bot. 31, 323–337.

Hardesty, B.D., Metcalfe, S., Westcott, D., 2009. Genetic inference provides insights into *Miconia calvescens* invasion within Australia. Proceedings of the International *Miconia* Conference.

John, G.P., Scoffoni, C., Buckley, T.N., Villar, R., Poorter, H., Sack, L., 2017. The anatomical and compositional basis of leaf mass per área. Ecol. Lett. 20,412–425.

Jud, W., Fischer, L., Canaval, E., Wohlfahrt, G., Tissier, A., and Hansell, A. 2016. Plant surface reactions: an opportunistic ozone defence mechanism impacting atmospheric chemistry. Atmospheric Chem. and Phys. 16, 277–292.

Keck, F., Rimet, F., Franc, A., Bouchez, A., 2016. Phylogenetic signal in diatom ecology: perspectives for aquatic ecosystems biomonitoring. Ecol Appl. 26, 861–872.

Kivimäenpää, M., Jonsson, A.M., Stjernquist, I., Sellden, G., Sutinen, S. 2004. The use of light and electron microscopy to assess the impact of ozone on Norway spruce needles. Environ. Pollut. 127, 441–453.

Koch, K., Ensikat, H.J., 2008. Hydrophobic coatings os plant surfaces: Epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. Micron 39, 759–772.

Lee, A.P., Upchurch, Jr.G., Murchie, E.H., Lomax, B.H., 2015. Leaf energy balance modelling as a tool to infer habitat preference in the early angiosperms. Proc R Soc B 282, 30–52.

Leite, F.T., 2017. Variação funcional em espécies de Rubiaceae em um gradiente ambiental na Floresta Atlântica. (Masters dissertation). Universidade Federal do Espirito Santo, Alegre, ES.

Li, P., Calatayud, V., Gao, F., Uddling, J., Feng, Z., 2016. Differences in ozone sensitivity among woody species are related to leaf morphology and antioxidant levels. Tree Physiol. 00, 1–12.

Li., S., Tosens, T., Harley, P.C., Jiang, Y., Kanagendran, A., Grosberg, M., Jaamets, K., Niinemets, Ü., 2018. Glandular trichomes as a barrier against atmospheric oxidative stress: Relationships with ozone uptake, leaf damage, and emission of LOX products across a diverse set of species. Plant Cell Environ. 41, 1263–1277.

Lima, M.E., Cordeiro, I., Moreno, P.R., 2011. Estrutura do componente arbóreo em Floresta Ombrófila Densa Montana no Parque Natural Municipal Nascentes de Paranapiacaba (PNMNP), Santo André, SP, Brasil. Hoehnea 38, 73–96.

Marques, A.R., Garcia, Q.S., Rezende, J.L.P., Fernandes, G.W., 2000. Variations in leaf characteristics of two species of *Miconia* in the Brazilian cerrado under different light intensities. Trop Ecol. 41, 47–60.

Medeiros, A.C., Loope, L.L., Conant, P., Mcelvaney, S., 1997. Status, Ecology, and Management of the Invasive Plant, *Miconia calvescens* DC (Melastomataceae) in the Hawaiian Islands. Museum Occasional Papers 48, 23–36. Mo, L., Ma, Z., Xu, Y., Sun, F., Lun, X., Liu, X., 2015. Assessing the Capacity of Plant Species to Accumulate Particulate Matter in Beijing, China. PLoS One 10 (10), e0140664.

Moura, B.B., Alves, E.S., 2014a. Climatic factors influence leaf structure and thereby affect the ozone sensitivity of *Ipomoea nil* 'Scarlet O'Hara'. Environ. Pollut. 194, 11–16.

Moura, B.B., Alves, E.S., De Souza, S.R., Domingos, M., Vollenweider, P., 2014b. Ozone phytotoxic potential with regard to fragments of the Atlantic semi-deciduous forest downwind of Sao Paulo, Brazil. Environ. Pollut. 192, 65–73.

Nakazato, R.K., Esposito, M.P., Cardoso-Gustavson, P., Bulbovas, P., Pedroso, A.N.V., Assis, P.I.L., Domingos, M., 2018. Efficiency of biomonitoring methods applying tropical bioindicator plants for assessing the phytoxicity of the air pollutants in SE, Brazil. Enviro Sci Pollut Res Int. 25, 19323–19337.

Niinemets, Ü., 1999. Research review Components of leaf dry mass per area - thickness and density- alter leaf photosynthetic capacity in reverse directions in woody plants. New Phytol.144, 35–47.

Nock, C.A., Vogt, R.J., Beisner, B.E., 2016. Functional Traits. In: L.S. John Wiley & Sons, Ltd: Chichester DOI: 10.1002/9780470015902.a0026282

Pääkkönen, E., Holopainen, T., Kärenlampi, L., 1997. Variation in ozone sensitivity among clones of *Betula pendula* and *Betula pubescens.* Environ. Pollut. 95, 37–44.

Paoletti, E., Grulke, N.E., 2005. Does living in elevated CO2 ameliorate tree response to ozone? A review on stomatal responses. Environ. Pollut. 137, 483–493.

Pedreira, G., Sousa, H.C., 2011. Comunidade arbórea de uma mancha florestal permanentemente alagada e sua vegetação adjacente em Ouro Preto, MG, Brasil. Ciência Florestal 21, 665–677.

Pereira, E.G., Oliva, M.A., Kuki, K.N., Cambraia, J., 2009. Photosynthetic changes and oxidative stress caused by iron ore dust deposition in tropical CAM tree *Clusia hilariana*. Trees 23, 277–285.

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I.J., Villar, R., 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta- analysis. New Phytol. 182, 565–588.

Poorter, L., Bongers, F., 2006. Leaf traits are good predictors of plant performance across 53 rain forest species. Ecology. 87, 1733–1743.

Roshchina, V.V. and Roshchina, V.D., 2003. In: Ozone and plant cell. Springer Science Business Media Dordrecht.

Saebo, A., Popek, R., Nawrot, B., Hanslin, H.M., Gawronska, H., Gawronski, S.W. 2012. Plant species differences in particulate matter accumulation on leaf surfaces. Sci Total Environ. 427/428, 347–354.

Sánchez-Lopéz, A.S., Carrillo-González, R., González-Chávez M.C.A., Rosas-Saito, G.H., Vangrosveld, J., 2015. Phytobarriers: Plants capture particles containing potentially toxic elements originating from mine tailings in semiarid regions. Environ. Pollut. 205, 33–42.

Sedio, B.E., Wright, S.J., Dick, C.W., 2012. Trait evolution and the coexistence of a species swarm in the tropical forest understorey. J. Ecol 100, 1183–1193.

Simons, E., Baranyai, E., Braun, M., Cserháti, C., Fábián, I., Tóthmérész, B., Elemental concentrations in deposited dust on leaves along an urbanization gradient. Sci. Total Environ. 490, 514–520.

Tanus, M.R., Pastore, M., Bianchini, R.S., Gomes, E.P.C., 2012. Florística e estrutura da comunidade arbóreo-arbustiva e o efeito de borda em trecho de Mata Atlântica no Parque Estadual das Fontes do Ipiranga, São Paulo, SP. Hoehnea 39, 157–168.

Tian, M., Yu, G., He, N., Hou, J., 2016. Leaf morphological and anatomical traits from tropical to temperate coniferous forest: Mechanisms and influencing factors. Sci Rep. 22; 6,19703.

Valladares, F., Wright. S.J., Lasso, E., Kitajima, K., Pearcy, R.W., 2000. Plastic phenotypic response to light of 16 congeneric shrubs from a panamanian rainforest. Ecology, 81, 1925–1936.

Violle, C., Navas, M.L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., Garnier, E.,

2007. Let the concept of trait be functional! Oikos 116, 882–892.

**Fig. S1.** Leaf surface SEM of the of some tree species from different remnants of the Atlantic Forest. A-M. Adaxial surface. N-Z. Abaxial surface. A-E. Smooth cuticle and glabrous surface (A, *Amaioua intermedia*; B, *Eugenia excelsa*; C, *Miconia cabucu; D, Ocotea paranapiacabensis*; E, *Psychotria vellosiana*). F. Smooth cuticle and simple non-glandular trichomes (*Machaerium villosum*). G. Smooth cuticle, stellar and simple non-glandular trichomes (*Croton floribundus*). H-I. Smooth cuticle, peltate glandular trichomes (H, *Eugenia ceraciflora*; I, *Myrsine umbellata*). J-K. Striated cuticle and glabrous surface (J, *Guarea kunthiana*; K, *Schinus terebenthifolia*). L. Striated cuticle and stellar non-glandular trichomes (*Astronium graveolens*). M. Striated cuticle and peltate glandular trichomes (*Psychotria suterella*). N-O. Smooth cuticle and glabrous surface (N, *Amaioua intermedia*; O, *Eugenia excelsa*). P, T. Stellar non-glandular trichomes densely distributed at different heights over the leaf surface (P, *Miconia cabucu*; T, *Croton floribundus*). Q. Simple non-glandular trichomes covered by a smooth cuticle (*Ocotea paranapiacabensis*); note the particles obstructing the stomatal pore. R. Smooth cuticle (*Psychotria vellosiana*). S. Deposition of epicuticular waxes in platelets and very long non-glandular trichomes dispersed over the leaf blade (*Machaerium villosum*). U. Smooth cuticle and peltate glandular trichomes (*Eugenia ceraciflora*). V. Striated cuticle and peltate glandular trichomes (*Myrsine umbellata*). W. Smooth cuticle, short and long non-glandular trichomes (*Guarea kunthiana*). X. Striated cuticle close to stomata, and a stellar non-glandular trichomes (*Astronium graveolens*). Y. Striated cuticle and a digitiform glandular trichome (*Schinus terebenthifolia*). Z. Smooth cuticle and simple non-glandular trichomes (*Psychotria vellosiana*); note the particles over the leaf blade and adhered to fungal hyphae (A, B, D, J, M– O, R, T, U, Z).

**Supplementary material**

## 1. Description of the leaf blade surface

**Table S1.** Median values (± standard deviations) of morphological leaf traits of the 12 pioneer (P) and 13 non-pioneer (NP) tree species sampled at the Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI). LA, leaf area; LMA, leaf mass area; LD, leaf density; DMC, dry matter concentration; RWC, relative water content. Distinct letters indicate significant differences in each morphological parameter among the tree species collected in the same remnant of Atlantic Forest ($p < 0.05$) The higher values are highlighted. One-way ANOVA on ranks (Kruskal Wallis test) followed by a multiple comparison (Dunn's method) were then applied to indicate significant for each morphological leaf trait between the tree species in each remnant of Atlantic Forest.

| **Tree Species (N= 4)** | **Funtional group** | **Morphological traits** | | | | |
|---|---|---|---|---|---|---|
| | | LA ($cm^2$) | LMA (mg $cm^{-2}$) | LD (mg $cm^3$) | DMC (mg $cm^{-3}$) | RWC (%) |
| | | MPP | | | | |
| *Guarea macrophylla* Vahl (Meliaceae) | NP | **94.24 (±14.97)a** | 0.007 (±0.001)ab | **0.26 (±0.044)b** | 1.92 (±0.27)ab | 76.9 (±6.29)ab |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | **98.06 (±13.19)a** | **0.02 (±0.0025)a** | **0.81 (±0.24)a** | 4.39 (±0.8)ab | **88.06 (±3.77)a** |
| *Myrsine umbellata* Mart. (Myrsinaceae) | P | 15.89 (±1.61)ab | **0.01 (±0.001)a** | 0.37 (±0.027)ab | **5.97 (±0.83)a** | **74.31 (±6.42)b** |
| *Ocotea paranapiacabensis* Coe-Teixeira (Lauraceae) | NP | 10.1 (±1.07)ab | 0.007 (±0.0009)ab | 0.38 (±0.06)ab | 1.32 (±0.22)bc | 77.65 (±7.03)ab |
| *Psychotria suterella* Müll. Arg. (Rubiaceae) | NP | **2.84 (±0.5)b** | **0.004 (±0.00014)b** | **0.23 (±0.011)b** | **0.67 (±0.064)c** | **72.03 (±7.67)b** |
| *Tibouchina pulchra* Cong (Melastomataceae) | P | 5.03 (±0.97)ab | 0.008 (±0.00077)ab | 0.35 (±0.034)ab | 2.2 (±0.21)ab | 83.17 (±3.25)ab |
| | | PEFI | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Alchornea sidifolia* Müll. Arg. (Euphorbiaceae) | P | 108.84 (±19.24)a | **0.005 (±0.00035)b** | **0.93 (±0.19)a** | 1.4 (±0.27)ab | 59.26 (±10.136)a |
| *Amaioua intermedia* Mart. ex Schult. & Schult.f. (Rubiaceae) | NP | 16.05 (±3.13)a | 0.009 (±0.00064)ab | 0.55 (±0.0084)ab | 1.54 (±0.08)ab | 63.57 (±2.791)a |
| *Eugenia excelsa* O. Berg (Myrtaceae) | NP | **3.92 (±1.07)b** | 0.006 (±0.00065)ab | 0.58 (±0.076)ab | **0.77 (±0.11)b** | 82.63 (±6.149)a |
| *Guarea macrophylla* Vahl (Meliaceae) | NP | 94.24 (±14.97)a | 0.008 (±0.00053)ab | **0.33 (±0.0218)b** | 2.04 (±0.19)ab | 72.41 (±1.89)a |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | 110.36 (±38.4)a | **0.01 (±0.0023)a** | **0.87 (±0.19)a** | **3.85 (±0.73)a** | 81.75 (±2.64)a |
| *Solanum granulosoleprosum* Dunal (Solanaceae) | P | 28.3 (±7.33)a | **0.005 (±0.0016)b** | 0.44 (±0.1)ab | **0.79 (±0.25)b** | 53.74 (±16.442)a |
| | | MSG | | | | |
| *Alchornea triplinervia* Müll. Arg. (Euphorbiaceae) | P | 108.84 (±19.24)ab | **0.005 (±0.00035)b** | 0.55 (±0.028)ab | **0.63 (±0.08)b** | **91.25 (±2.16)a** |
| *Astronium graveolens* Jarcq. (Anacardiaceae) | NP | 95.21 (±23.21)abc | **0.006 (±0.0011)b** | 0.65 (±0.022)ab | **0.65 (±0.07)b** | 86.46 (±0.74)ab |
| *Croton floribundus* Spreng. (Euphorbiaceae) | P | 47.59 (±7.92)bc | 0.007 (±0.0014)ab | 0.57 (±0.081)ab | 1.15 (±0.29)ab | 81.25 (±9.32)ab |
| *Guarea kunthiana* A. Juss. (Meliaceae) | NP | **818.71 (±85.61)a** | **0.01 (±0.002)a** | **0.37 (±0.072)b** | **4.73 (±0.64)a** | **53.29 (±0.94)b** |
| *Ocotea beulahiae* J.B. Baitello (Lauraceae) | NP | **19.64 (±6.57)c** | **0.01 (±0.001)ab** | 0.5 (±0.096)ab | 2.21 (±0.18)ab | 68.59 (±14.78)ab |
| *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Fabaceae) | P | 60.07 (±10.73)abc | 0.009 (±0.0015)ab | **0.77 (±0.045)a** | 1.18 (±0.33)ab | _ |
| | | PEI | | | | |
| *Drimys brasiliensis* Miers (Winteraceae) | NP | 9.23 (±0.82)ab | 0.009 (±0.0009)abc | **0.27 (±0.023)c** | **3.36 (±0.46)a** | **84.52 (±2.53)a** |
| *Eremanthus erytropappus* (DC.) MecLeisch (Asteraceae) | P | 10.98 (±0.8)ab | 0.01 (±0.0007)abc | 0.67 (±0.047)ab | 1.6 (±0.14)ab | 61.42 (±4.77)ab |
| *Eugenia cerasiflora* Miq. (Myrtaceae) | NP | **3.19 (±0.4)b** | 0.009 (±0.0016)abc | 0.5 (±0.069)abc | 2.05 (±0.5)ab | **49.17 (±5.01)b** |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Machaerium villosum* Vogel (Fabaceae) | NP | **54.01 (±20.11)a** | 0.008 (±0.0014)bc | 0.48 (±0.064)abc | 1.49 (±0.34)ab | **41.22 (±6.3)b** |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | **50.85 (±9.97)a** | **0.01 (±0.0033)a** | **0.89 (±0.21)a** | **3.74 (±0.24)a** | 61.22 (±4.53)ab |
| *Psychotria vellosiana* Benth (Rubiaceae) | NP | **2.21 (±0.79)b** | **0.005 (±0.0014)c** | **0.33 (±0.091)bc** | **0.85 (±0.23)b** | 76.7 (±0.95)ab |
| *Schinus terebinthifolia* Raddi (Anacardiaceae) | P | 17.67 (±3.93)ab | 0.01 (±0.001)abc | 0.48 (±0.048)abc | 2.69 (±0.24)ab | **83.90 (±3.81)a** |

**Table S2.** Median values (± standard deviations) for morphological leaf traits of the 12 pioneer (P) and 13 non-pioneer (NP) tree species sampled at the Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI). LT, leaf thickness; PP, palisade parenchyma thickness; SP, spongy parenchyma thickness; IS, intercellular space; SD, stomatal density of the adaxial surface; SD, stomatal density of the abaxial surface; TD, trichome density of the adaxial surface; TD, trichome density of the abaxial surface. Distinct letters indicate significant differences among the tree species in each anatomical parameter and remnants of Atlantic Forest ($p < 0.05$). Highest values are shown in bold and the lowest values in gray. One-way ANOVA on ranks (Kruskal Wallis test) followed by a multiple comparison (Dunn's method) were then applied to indicate significant for each anatomical leaf trait between the tree species in each remnant of Atlantic Forest.

| | | **Anatomical traits** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Tree Species (N= 4)** | **Funtional group** | LT (μm) | PP (μm) | SP (μm) | IS (%) | Adaxial surface SD (N/mm$^2$) | Abaxial surface SD (N/mm$^2$) | Adaxial surface TD (N/mm$^2$) | Abaxial surface TD (N/mm$^2$) |
| | | | | MPP | | | | | |
| *Guarea macrophylla* Vahl (Meliaceae) | NP | 258.38 (±12.81)ab | **44.44 (±2.61)c** | **182.4 (±10.98)ab** | 18.15 (±5.54)ab | 0 (±0)a | 4.66 (±0.78)bc | 2 (±0.27)ab | 6.16 (±1.14)a |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | 234.2 (±36.4)ab | **135.82 (±35.22)a** | **57.9 (±3.96)c** | **4.47 (±3.22)b** | 0 (±0)a | 24.5 (±6.8)ab | **0 (±0)b** | 52.08 (±1.5)a |
| *Myrsine umbellata* Mart. (Myrsinaceae) | P | **412.41 (±28.99)a** | **90.73 (±17.54)ab** | **252.4 (±19.27)a** | **32.76 (±7.74)a** | 0 (±0)a | 6.16 (±0.87)abc | 0,667 (±0.33)ab | **1 (±0.13)b** |
| *Ocotea paranapiacabensis* Coe- | NP | **174.38 (±19.79)b** | 55.24 (±7.58)abc | 90.2 (±13.7)abc | 15.19 (±4.11)b | 0 (±0)a | 9.83 (±1.41)abc | **0 (±0)b** | **0.66 (±0.41)b** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Teixeira (Lauraceae) | | | | | | | | | |
| *Psychotria suterella* Müll. Arg. (Rubiaceae) | NP | **167.71 (±10.83)b** | **50.94 (±2.88)bc** | **65.07 (±8.12)bc** | 16.82 (±6.85)ab | 0 (±0)a | **3 (±0.9)c** | **0 (±0)b** | 5.33 (±1.15)a |
| *Tibouchina pulchra* Cong (Melastomataceae) | P | 249.43 (±10.08)ab | 89.67 (±5.85)abc | 120.4 (±8.35)abc | **4.47 (±1.28)b** | 0 (±0)a | **28.66 (±2.8)a** | **4.66 (±0.167)a** | 6.83 (±0.9)a |
| PEFI | | | | | | | | | |
| *Alchornea sidifolia* Müll. Arg. (Euphorbiaceae) | P | **119.76 (±24.21)b** | 54.45 (±17.54)ab | **39.37 (±3.76)b** | 13.85 (±3.24)ab | 0 (±0)a | 7.83 (±0.31)ab | 1 (±0.41)ab | 11 (±3.19)ab |
| *Amaioua intermedia* Mart. ex Schult. & Schult.f. (Rubiaceae) | NP | 164.8 (±4.14)ab | 38.76 (±14.97)ab | 86.52 (±5.04)ab | 20.83 (±10.1)ab | 0 (±0)a | 7 (±1.01)ab | 0.33 (±0.5)ab | 3 (±1.01)ab |
| *Eugenia excelsa* O. Berg (Myrtaceae) | NP | **121.28 (±9.83)b** | **30.38 (±5.32)b** | 70.76 (± 6.49)ab | **4.35 (±0.35)b** | 0 (±0)a | **21.33 (±0.73)a** | **0 (±0)b** | **0 (±0)b** |
| *Guarea macrophylla* Vahl (Meliaceae) | NP | **254.07 (±11.83)a** | 44.12 (±3.55)ab | **169.15 (±3.83)a** | **26.69 (±1.79)a** | 0 (±0)a | **4 (±1.08)b** | 1.66 (±0.41)ab | 6.16 (±1.05)ab |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | 199.09 (±18.8)ab | **102.08 (±24.35)a** | 64.04 (±7.68)ab | 9.01 (±1.91)ab | 0 (±0)a | **25.16 (±4.45)a** | **0 (±0)b** | **38.66 (±11.43)a** |
| *Solanum granulosoleprosum* Dunal (Solanaceae) | P | 128.56 (±12.46)ab | 61.27 (±8.21)ab | **44.75 (±4.8)b** | 15.81 (±7.28)ab | 1.5 (±0.69)a | 16.66 (±4.79)ab | **27.16 (±10.81)a** | **24 (±2.49)a** |
| | | | | MSG | | | | | |
| *Alchornea triplinervia* Müll. Arg. (Euphorbiaceae) | P | **106.3 (±8.31)b** | 47.55 (±4.06)a | **37.03 (±5.3)b** | 35.02 (±14.84)a | 1.5 (±0.63)a | 7.33 (±0.57)ab | 0.66 (±0.68)ab | 5.33 (±2.78)ab |
| *Astronium graveolens* Jarcq. (Anacardiaceae) | NP | **101.54 (±4.484)b** | 42.77 (±2.44)a | **35.84 (±3.06)b** | 18.99 (±14.65)a | 1. 33 (±0.33)a | 8.33 (±0.57)ab | 1.66 (±0.50)ab | 4 (±1.01)ab |
| *Croton floribundus* Spreng. (Euphorbiaceae) | P | 146.68 (±13.39)ab | 62.9 (±7.89)a | 52.23 (±7.17)ab | 16.36 (±11.28)a | 0 (±0)a | **10.33 (±1.87)a** | **4 (±0.54)a** | **19.5 (±0.68)a** |
| *Guarea kunthiana* A. Juss. (Meliaceae) | NP | **361.6 (±27.46)a** | 62.86 (±13.79)a | **267.84 (±21.89)a** | 32.91 (±3.51)a | 0 (±0)a | **6.66 (±0.5)b** | **0.33 (±0.16)b** | **16.5 (±7.6)a** |
| *Ocotea beulahiae* J.B. Baitello (Lauraceae) | NP | 209.11 (±21.17)ab | 47.24 (±2.09)a | 128.08 (±20.25)ab | 18.33 (±7.63)a | 0 (±0)a | 7.5 (±1.19)ab | **0.16 (±0.63)b** | **0.16 (±0.19)b** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Fabaceae) | P | 123.1 (±12)ab | 49.73 (±8.74)a | 47.78 (±3.81)ab | 15.24 (±9.62)a | 0 (±0)a | **11 (±1.79)a** | 1 (±0.73)ab | 2.83 (±5.96)ab |
| PEI | | | | | | | | | |
| *Drimys brasiliensis* Miers (Winteraceae) | NP | **362.98 (±20.05)a** | 120.8 (±6.91)ab | **184.84 (±12.99)a** | **20.69 (±2.76)a** | 0 (±0)a | **6.83 (±1.12)b** | 1.83(±0,56)ab | **0 (±1.826)b** |
| *Eremanthus erytropappus* (DC.) MecLeisch (Asteraceae) | P | 238.51 (±5.65)ab | **131.29 (±7.73)a** | 63.42 (±1.85)abc | 9.27 (±1.47)ab | 0 (±0)a | 9.16 (±0.87)ab | **11.33(±1.82)a** | **0 (±0)b** |
| *Eugenia cerasiflora* Miq. (Myrtaceae) | NP | 223.61 (±25.58)abc | 84.81 (±25.07)abc | 96.8 (±10.24)ab | 16.89 (±1.75)ab | 0 (±0)a | 12.33 (±0.95)ab | 2.33(±0.63)ab | 2 (±0.577)ab |
| *Machaerium villosum* Vogel (Fabaceae) | NP | 165.68 (±14.9)bc | 68.41 (±12.68)bc | 75.06 (±4.7)abc | 13.62 (±2.294)ab | 0 (±0)a | **13.58 (±1.27)a** | **13.5(±2.88)a** | **9.83 (±1.402)a** |
| *Miconia cabucu* Hoehne (Melastomataceae) | P | 202.62 (±19.42)abc | 107.16 (±18.13)abc | **44.19 (±1.43)c** | **4.957 (±3.85)b** | 0 (±0)a | **35.66 (±6.53)a** | **0(±0)b** | **37.83 (±6.274)a** |
| *Psychotria vellosiana* Benth (Rubiaceae) | NP | **153.54 (±5.61)c** | **46.41 (±2.76)c** | 56.21 (±6.48)bc | 21.25 (±12.81)ab | 0 (±0)a | 8.333 (±1.16)ab | **0(±0)b** | 5.66 (±1.826)ab |
| *Schinus terebinthifolia* Raddi (Anacardiaceae) | P | 203.63 (±6.09)abc | 102.59 (±5.1)abc | 62.18 (±5.93)abc | 11.26 (±3.04)ab | 0 (±0)a | 9.33 (±1.13)ab | 2.33(±1.27)ab | 2.33 (±0.577)ab |

Capítulo 2

# Tolerance level to oxidative stress is validated in tropical tree species by using foliar microscopic markers[6]

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[6] Capítulo a ser submetido ao periódico Ecological Indcators (QUALIS A2; IF 4.4).

**Abstract**

We previously revealed that morpho-anatomical leaf traits are predictors of the potential level of tolerance or susceptibility in native tree species to environmental stressors, such as the combination of air pollutants that act synergistically with natural environmental stressors (variations and climatic abnormalities) in the Atlantic Forest. Here, we hypothesized that species with lower potential of tolerance show a greater variety of microscopic markers (i.e. structural changes) in their seemingly healthy leaf blades in response to oxidative stress than species with higher potential of tolerance. In this regard, tree species with distinct potential of tolerance to oxidative stress (tolerant, intermediate and sensitive species) were sampled in four remanants of Atlantic Forest in SE Brazil. Histochemical tests to detect the microscopic markers were applied in seemingly healthy leaf blades of species. Overall, the results indicated that potential intermediate tolerant and sensitive species to environmental stress showed greater variety and frequency of microscopic markers in the mesophyll (wart-like protrusion, invagination of plasma membrane, vacuome and partial/total plasmolysed cell with condensation of cellular content and disrupted cells). Conversely, tolerant species showed microscopic markers that are indicators of increased resistance against oxidative stress, such as hypertrophy of mesophyll cells and accumulation of phenolic glycosides in the apoplast that increase the cellular defense against reactive oxygen species. The accumulation of phenolic glycosides in the apoplast is a new microscopic marker identified in leaves of tropical tolerant species. Foliar microscopic markers allowed the assessment and validation of the tolerance level of species from Atlantic Forest to oxidative stress.

**Keywords:** air pollutants, Atlantic Forest, phenolic glycosides, sensitive tree species, structural responses, tolerant species-specific markers

## 1. Introduction

Specific responses, such as foliar visible injury that appears in sensitive tree species, have long been evaluated for estimating the effects of ozone (among other oxidative pressures) on forest ecosystems in the Northern Hemisphere (Schaub et al., 2016). Many authors have validated the occurrence of visible leaf injury by means of microscopic markers. These symptoms can be linked to different physiological reactions, including: hypersensitive responses (Vollenweider et al., 2003; Bussotti et al., 2005; Gravano et al., 2004; Paoletti et al., 2009; Guerrero et al., 2013; Fernandes et al., 2016; Moura et al., 2011, 2018); acceleration of plant senescence (Paoletti et al., 2009; Vollenweider et al., 2013; Fernandes et al., 2019); oxidative burst in the apoplast (Günthardt-Goerg et al., 1997, 2000; Vollenweider et al., 2003; Günthardt-Goerg and Vollenweider, 2007; Paoletti et al., 2009; Pedroso et al., 2016; Moura et al., 2018) and in other cell compartments (Vollenweider et al., 2003; Busssotti et al., 2005; Moura et al., 2018), besides defensive and repair reactions (Orendovici et al., 2003; Bussotti et al. 2005; Guerrero et al., 2013; Alves et al., 2016; ; Fernandes et al., 2016; Moura et al., 2018). However, the strategies adopted by tropical plants to resist (or not) oxidative stress are still virtually unknown, particularly in the Brazilian Atlantic forest, and their search becomes a challenge due to several particular reasons.

In contrast to temperate forests, the effects of complex mixtures of air pollutants and climatic oscillations on plants may restrict the assessment of specific plant responses to oxidative pressures in the Atlantic forest. These effects result in an indiscriminately increased production of reactive oxygen species (ROS) in plant cells, which may cause similar visible or microscopic leaf damage. This similarity of symptoms is linked to the high tree species diversity observed in this biome (Moura et al., 2014; Cardoso-Gustavson et al., 2015; Domingos et al., 2015; Brandão et al., 2017;

Nakazato et al., 2018). In addition, the Atlantic Forest canopy remains with leaves during the whole the year in most cases, whereas the foliage turnover is highly species-specific (Moura et al., 2018).

We may also assume that the combination of air pollutants acts synergistically with other environmental stressors in the Atlantic Forest species. This synergism causes defense responses to oxidative stress and irreversible oxidative cell damage even in leaf blades without visible injuries (Kivimäenpää et al., 2003; Vollenweider et al., 2003), as observed in the native pioneer tree species *Tibouchina pulchra* (Melastomataceae) by Pedroso et al. (2016). The structural leaf changes (referred here as microscopic markers) in this tree species are similar to those recorded in forests located in other regions of the world (Pedroso et al., 2016; Moura et al., 2018). Interestingly, other tropical species can also respond to environmental stresses with specific structural markers related to an increase in cellular defenses, such as a vacuolar accumulation of mucilage-like polyssacharides (Moura et al., 2018) or hydrolysable tannins (Fernandes et al., 2016), and the accumulation of phenolic compounds in the apoplast that is not usually described in temperate species (Fernandes, 2015).

The morpho-anatomical leaf traits can explain the different levels of tress species tolerance to oxidative stress (Bussotti, 2008). In chapter 1, we showed that morpho-anatomical leaf traits are predictors of the potential level of tolerance or susceptibility in native forest species to multiple environmental conditions (air pollutants and climatic changes) and groups of species with distinct potential of tolerance were identified. In addition, we also noticed that the potential of tolerance varied between functional groups (pioneer *vs* non-pioneer species). Pioneer species have morpho-anatomical leaf traits together with physiological leaf traits (Brandão et al., 2017, Esposito et al., 2018) that confer a greater potential of tolerance than non-pioneer

species. By assuming the veracity of the former sentence, here we tested the hypothesis that species with lower potential of tolerance to environmental stresses show a greater variety of microscopic markers in their seemingly healthy leaf blades in response to oxidative stress than species with higher potential of tolerance.

In this regard, our objectives were: (1) to search for structural microscopic markers in three groups of species with distinct potential of tolerance to oxidative stress by environmental stressors (tolerant, intermediary and sensitive species, previously defined in chapter 1) and (2) to validate the tolerance level to oxidative stress in each group based on microscopic markers.

The results obtained here confirmed the hypothesis because the microscopic responses were widely detected among the species with some degree of sensitivity to oxidative stress. In addition, we also proposed a new microscopic marker for tolerant species.

**2. Material and methods**

The tree species selected for this study were sampled in four remaining Atlantic Forest, in SE Brazil: Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), ecological area of relevant interest Mata de Santa Genebra (MSG) (all included in conservation units located in the state of São Paulo), and State Park Itacolomi (PEI) (included in conservation unit located in the state of Minas Gerais). All these forest remnants differ in forest physiognomy, species composition, climatic characteristics and emission sources of pollution (see Chapter 1 for more details).

The choice of native tree species was based on their (1) potential tolerance level to oxidative stress induced by environmental stressors, which were established in chapter 1 by several morpho-anatomical leaf traits, as summarized in the Table 1.

**Table 1.** Potential tolerance level to oxidative stress imposed by environmental stressors, based on morpho-anatomical leaf traits (according to the results presented in chapter 1) of tree species sampled at the Municipal Park Paranapiacaba (MPP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park Itacolomi (PEI). Leaf mass area (LMA); leaf density (LD); dry matter concentration (DMC); leaf thickness (LT); palisade parenchyma thickness (PP); spongy parenchyma thickness (SP); intercellular space (IS); abaxial stomatal density (SD); abaxial trichomes density (TD).

| **Tree species** | **Atlantic Forest remnant** | **Potential tolerance level** | **Morpho-anatomical traits** |
|---|---|---|---|
| *Miconia cabucu* Hoehne (Melastomataceae) | MPP, PEFI and PEI | Tolerant | High LMA, DMC, PP, TD and SD |
| *Schinus terebinthifolia* Raddi (Anacardiaceae) | PEI | | |
| *Alchornea triplinervia* Müll. Arg. (Euphorbiaceae) | MSG | Intermidiate | High LD and low LT and SP |
| *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Fabaceae) | MSG | | |
| *Solanum granulosoleprosum* Dunal (Solanaceae) | PEFI | | |
| *Amaioua intermedia* Mart. ex Schult. & Schult.f. (Rubiaceae) | PEFI | Sensitive | Low LMA, DMC, PP, TD and SD |
| *Guarea macrophylla* Vahl (Meliaceae) | MPP and PEFI | | |
| *Ocotea paranapiacabensis* Coe-Teixeira (Lauraceae) | MPP | | |
| *Drimys brasiliensis* Miers (Winteraceae) | PEI | | High LT and SP and low LD |
| *Guarea kunthiana* A. Juss. (Meliaceae) | MSG | | |
| *Myrsine umbellata* Mart. (Myrsinaceae) | MPP | | |

The leaf collection was carried out in the wet (January to February / 2016) and dry periods (July to August / 2016). Two sunny leaves from three specimens per species (N = 6 leaves per species) were collected in each forest remnant to identify the presence/absence of microscopic markers of oxidative stress in the leaf blades.

### 2.1. Assessment to microscopic markers

Fragments (approx. 1 $cm^2$) of the median region of the leaf blade were fixed in 2.5 % glutaraldehyde buffered at pH 7.0 with 0.067 M Sorensen phosphate buffer. After

that, samples were fully evacuated and stored at 4 °C. In the laboratory, the fixed samples were included in Technovit 7100 historesin and semi-thin 3 μm thickness sections were obtained using a rotary microtome Leica (RM2245). The samples were submitted to histochemical tests to detect the microscopic markers as described in previous studies (Table 2). All the observations were performed in a light microscopy (Olympus BX53), equipped with an image capture system (ImagePro-express software 6.3). The whole leaf section in each slide was systematically evaluated in order to locate microscopic markers.

Finally, the frequency of a specific microscopic marker found in the leaf blade of each species was calculated by estimating the percentage of samples in which it was found at least once.

**Table 2.** Staining methods applied on leaf samples, microscopic markers and stress agents.

| Stain | Reference Stain | Target compound | Color in transmitted light | Excitation (nm) | Microscopic marker | Stress agent | Reference |
|---|---|---|---|---|---|---|---|
| toluidine blue | Feder and O'Brien, 1968 | metachromatic | blue/green | – | wart-like protrusions on cell wall | experimental heavy metals and acidic rainwater exposure; experimental $O_3$ exposure; ambient $O_3$ | Günthardt-Goerg et al., 1997; Bussotti et al., 2005; Günthardt-Goerg and Vollenweider, 2007; Hermle et al., 2007; Calatayud et al., 2011; Guerreiro et al., 2013; Moura et al., 2018 |
| | | | | | invagination of the plasma membrane | ambient $O_3$; multiple environmental conditions (air pollutants: $NO_2$, SO2 and $O_3$) + marked seasonal climate | Tresmondi and Alves, 2011; Pedroso et al., 2016 |

| | | |
|---|---|---|
| partial/ total plasmolysis of cells and sometimes cell content disruption | experimental Cu / Zn / Cd exposure; experimental $O_3$ exposure;; experimental $O_3$ exposure + drought; ambient $O_3$ + light ; environment with mix of pollutants ($NO_2$, $SO_2$ and $O_3$) + marked seasonal climate | Pääkkönen et al., 1998; Vollenweider et al., 2003; André et al., 2006; Vollenweider et al., 2006; Günthardt-Goerg and Vollenweider, 2007; Bussotti et al., 2005; Guerreiro et al., 2013; Pedroso and Alves, 2015; Fernandes et a., 2016, 2019; Pedroso et al., 2016; Moura et al., 2018 |
| accumulation of phenolics in vacuole | experimental heavy metals and acidic rainwater exposure; ambient $O_{3;}$ ambient $O_3$ + light; experimental O3 exposure + drought | Pääkkönen et al., 1998; Vollenweider et al., 2003; André et al., 2006; Her Tresmondi and Alves, 2011; Fernandes et al., 2016 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | vacuome[1] | environment with mix of pollutants (NO2, SO2 and O3) + marked seasonal climate | Pedroso et al., 2019 |
| | | | | | | hypertrophy[2] | acid rain; experimental $O_3$ exposure | Fink, 1999; Silva et al., 2005; Sant'Anna-Santos et al., 2006; Fernandes et al., 2019 |
| | | | | | | hyperplasia[3] | acid rain; experimental $O_3$ exposure | Silva et al., 2005; Sant'Anna-Santos et al., 2006; Fernandes et al., 2019 |
| p-phenylenediamine | Kivimäenpää et al., 2004 | lipids | dark gray | – | | darkening cloroplast | ambient $O_3$; Cd | Kivimäenpää et al., 2004; Vollenweider et al., 2006 |
| | | | | | | accumulation of phenolics in vacuole | experimental heavy metals and acidic rainwater exposure ; experimental $O_3$ exposure + eleveted temperature; experimental Cd exposure | Vollenweider et al., 2006; Hermle et al., 2007; Kivimäenpää et al., 2014; |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PARS reaction | Gahan 1984 | polysaccharides | pink | – | accumulation of plastoglobules into cloroplast | experimental heavy metals and acidic rainwater exposure; experimental $O_3$ exposure; ambient O3 | Hermle et al., 2007; Paoletti et al., 2009; Moura et al., 2018 |
| | | | | | cell wall thickening | experimental $O_3$ exposure; ambient $O_3$ | Bussotti et al., 2005; Vollenweider et al., 2013 |
| | | | | | polissaccharides wart-like protrusion | experimental $O_3$ exposure; ambient O3 | Moura et al., 2018 |
| | | | | | oxidation of wart-like protrusions | experimental O3 exposure | |
| | | | | | phenolic glicosydes in vacuole and apoplast | ambient $O_3$ + light | Fernandes, 2015 |
| | | | | | increase/deacrese of starch grains within cloroplast | experimental $O_3$ exposure; ambient $O_3$; experimental Cd exposure | Bussotti et al., 2005; Volenweider et al., 2006; Moura et al., 2018; Fernandes et al., 2019 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Aniline Blue | Gerlach 1984 | calloses | – | 340-380 | cellulose cell wall thickening | experimental $O_3$ exposure; ambient $O_3$; ambient O3 + light | Gravano et al., 2003, Gravano et al., 2004, Bussotti et al., 2005; Calatayud et al., 2011 Moura et al., 2018 |
| Calcofluor White | Munch 1989 | cellulose | – | 340-380 | callose cell wall thickening | experimental $O_3$ exposure; experimental $O_3$ exposure + drought; ambient $O_3$; ambient $O_3$ + light | Pääkkönen et al., 1998; Günthardt-Goerg et al., 1997; Vollenweider et al., 2003; Gravano et al., 2004; Bussotti et al., 2005; Moura et al., 2018. |

[1] fragmentation of the central vacuole in numerous small vesicles;

[2] increase in cell size;

[3] increase in the number of cell layers.

## 3. Results

### 3.1. Summary of structural leaf traits from species with distinct potential tolerance levels

The structural traits of cross-sectioned asymptomatic leaf blades of tree species with distinct potential tolerance levels to oxidative stress are shown in Table 3 and Fig.1. All species showed uniseriated epidermal cells, most of them were hipostomatic (except the anphistomatic *S. granulosoleprosum*) and had thick cuticle (except *A. intermedia, A. triplinervia, O. paranapiacabensis* and *S. terebenthifolia*). The main structural differences among potential tolerant, intermediate and sensitive groups of species were observed in the mesophyll (Table 3; Fig. 1).

The potential tolerant species showed 2-3 cell layers of palisade parenchyma and 3-5 cell layers of the spongy parenchyma. They also showed a great accumulation of polyphenols within vacuoles, and a compact mesophyll containing dispersed secretory structures (Table 3; Fig.1 A-B).

The intermediate tolerant species showed 1-2 layers of palisade parenchyma and 3-3 layers of spongy parenchyma, vacuole containing polyphenols and compact mesophyll (Table 3; Fig.1 C-E). Secretory structures were observed only in *P. gonoacantha* (Table 3; Fig. 1D).

The potential sensitive species showed 1-3 layers of palisade parenchyma and 3-12 layers of spongy parenchyma. *G. kunthiana, G. macrophylla* and *M. umbellate* showed accumulation of vacuolar polyphenols, whereas *A. intermedia*, *D. brasiliensis* and *O. paranapiacabensis* showed hyaline vacuoles. The mesophyll was characterized by pronounced intercellular spaces, and bear secretory structures dispersed in mesophyll, except *A. intermedia* (Table 3; Fig.1 F-K). Among all species groups, only *D. brasiliensis, G. macrophylla* and *M. umbellata* did not showed crystal idioblasts

(Table 3; Fig. 1G, I-J).

### 3.2. Microscopic markers in leaf tissues of species with distinct potential tolerance levels

The potential tolerant species to oxidative stress showed changes in cell wall and apoplast. *M. cabucu* showed wart-like protrusions on cell wall protruding into the inter-cellular space (Fig. 2A). The oxidation of compounds conferred brownish coloration in the apoplast (the same observed within the vacuole) in *M. cabucu* (Fig. 2B). In, addition, a positive PARS reaction in the apoplast indicative of alpha 1,2-glicol linkage was evidenced (Fig. 2B). This linkage is observed in the structure of some polysaccharides, but it also constitutes the structure of glycosylated derivatives of flavonoids. Callose or cellulose was not evidenced in this same region. The compounds embedded in apoplast were specific of tolerant species. An increase in spongy parenchyma cell size (hypertrophy) was visualized in *S. terebenthifolia* (Fig. 2D).

The species categorized as intermediate tolerant bearded wart-like protrusions (Fig. 3A-D). *A. triplinervia* showed invagination of plasma membrane (protoplast change), restricting the cell space of the palisade parenchyma (Fig. 3E). Discrete groups of parenchyma cells showed total/or partial plasmolysed cells, disorganized and dense content (Fig. 3F-H). Some plasmolysed cells also showed a disrupted cell content, as observed in *Solanum granulosoleprosum* (Fig. 3F).

The potential sensitive species showed the same markers described for intermediate tolerant species. The species showed plasmolyzed parenchymal cells (Fig. 4A-D), wart-like protrusion (Fig. 4E-I) and protoplast changes (*D. brasiliensis* and *M. umbellate,* Fig. 4J-K). However, the plasmolysed parenchyma cells in these species occupied a greater extension of the leaf blade (particularly in *A. intermedia*, Fig 4.A) than observed in the leaf blades of intermediate species (Fig 4.A *vs* Fig. 3 F-H). The

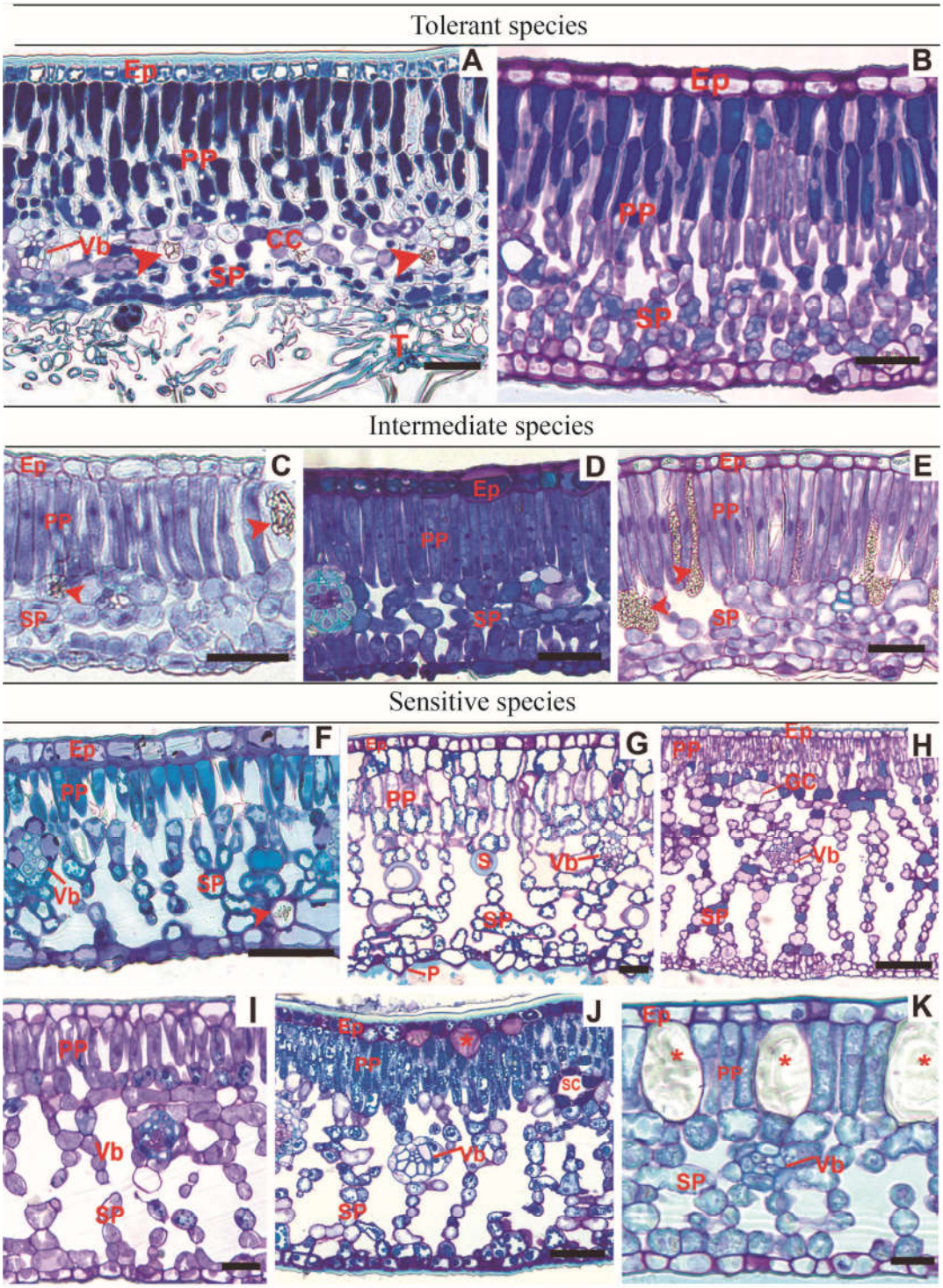
fragmentation of the central vacuole in numerous small vesicles (vacuome of palisade parenchyma cells) was a sensitive species-specific marker, visualized in *G. macrophylla* and *A. intermedia* (Fig. 4L-M). *D. brasiliensis* showed hypertrophy of spongy parenchyma cells (Fig. 4N), which was a common marker between tolerant and sensitive species (Fig. 2D *vs*. 4N).

The highest percentage and greater diversity of microscopic markers were found in leaves sampled during the wet period than during the dry period (Table 4). The wart-like protrusions occurred in potential tolerant group only in the wet season (47%; Table 4). In contrast, intermediate and sensitive groups of species showed wart-like protrusions in both wet (33-17% and 83-33%, respectively) and dry periods (17-50% and 17-50%, respectively) (Table 4). The wart-like protrusions appeared more frequently in the potential sensitive group (e.g. this microscopic marker was found in 83% of leaf blade sections of *D. brasiliensis* analyzed) (Table 4). The intermediate and sensitive groups showed plasmolyzed cells (17%-50% and 17-33%, respectively) in both periods (Table 4). In addition, intermediate and sensitive species showed protoplast changes in both wet (50% and 17-33%, respectively) and dry periods (33% and 17%, respectively). The intermediate group presented the highest percentage of plasmolyzed cells (*A. triplinervia* and *S. granulosoleprosum* showed 50%) and protoplast changes (*A. triplinervia* showed 50%). The frequency of vacuome in palisade parenchyma cells (observed only in the potential sensitive group) was higher in dry (*A. intermedia* showed 17%) than in wet period (*G. macrophylla* showed 8%). Hypertrophy appeared only in leaves collected in the wet period, with the same frequency in tolerant and sensitive groups (17%) (Table 4). The potential tolerant group showed 35% of samples with compounds embedded in the apoplast during the wet period (Table 4).

**Table 3.** Structural leaf traits in tree group of species with distinct potential of tolerance to oxidative stress by environmental stressors sampled in different remnants of the Atlantic Forest. + (present); - (absent)

| Species | Potential of tolerance to oxidative stress | Mesophyll | | | | | Figures |
|---|---|---|---|---|---|---|---|
| | | Layers of palisade parenchyma cells | Layers of spongy parenchyma cell | Vacuolar polyphenols | Crystal idioblasts | Secretory structures | |
| *M. cabucu* | Tolerant | 3 | 1-4; 1 intermediate cells | + | + | + | 1. A |
| *S. terebinthifolius* | Tolerant | 2-3 | 4-5 | + | + | + | 1. B |
| *A. triplinervia* | Intermidiate | 1 | 4 | + | + | – | 1. C |
| *P. gonoacantha* | Intermidiate | 2 | 4-5 | + | + | + | 1. D |
| *S. granulosoleprosum* | Intermidiate | 1 | 3 | + | + | – | 1. E |
| *A. intermedia* | Sensitive | 1-2 | 3-4 | – | + | – | 1. F |
| *D. brasiliensis* | Sensitive | 3 | 6-8; sclereids | – | – | + | 1. G |
| *G. kunthiana* | Sensitive | 2 | 8-12 | + | + | + | 1. H |
| *G. macrophylla* | Sensitive | 1 | 8-10 | + | – | – | 1. I |
| *M. umbellata* | Sensitive | 1 | 6-8 | + | – | + | 1. J |
| *O. paranapiacabensis* | Sensitive | 1 | 4-6 | – | + | + | 1. K |

**Fig. 1**. Structural traits in cross sections of asymptomatic leaf blades of potential potential tolerant, intermediate and sensitive species to oxidative stress sampled in different remnants of the Atlantic Forest. A. *Miconia cabucu.* B. *Schinus terebenthifolia.* C. *Alchornea triplinervia.* D. *Piptadenia gonoacantha.* E. *Solanum granulosoleprosum.* F. *Amaioua intermedia.* G. *Drimys brasiliensis.*H. *Guarea kunthiana.* I. *Guarea macrophylla.* J. *Myrsine umbellata.* K *Ocotea paranapiacabensis.* CC= collector cells; SC= secretory canal; S= sclereids; Ep= Epidermalcells; Vb= vascular bundles; GC= glandular cell; PP=Palisade parenchyma cells; SP= Spongy parenchyma cell; P = papillose *epidermis*; arrow head = crystalline idioblast; asterisk = secretory idioblast. Bars= 20 µm


Tolerant species
A
Ep
PP
Vb
CC
SP
T
B
Ep
PP
SP
Intermediate species
C
Ep
PP
SP
D
Ep
PP
SP
E
Ep
PP
SP
Sensitive species
F
Ep
PP
Vb
SP
G
Ep
PP
S
Vb
SP
P
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PP
CC
Vb
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J
Ep
PP
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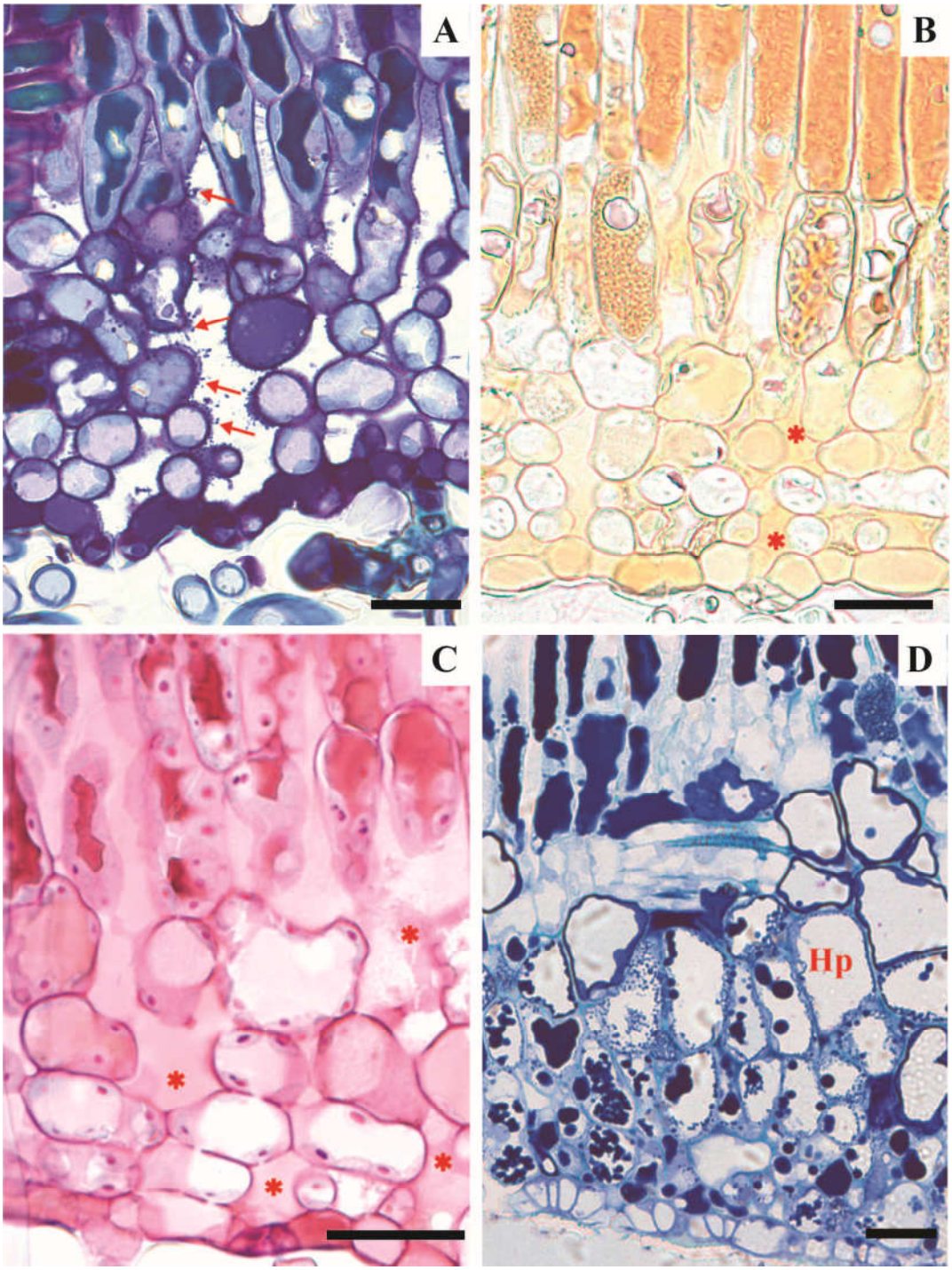
SP
K
Ep
PP
Vb
SP

**Fig. 2.** Microscopic markers in the mesophyll cells of potential tolerant species to oxidative stress from different remnants of the Atlantic Forest. A. Wart-like protrusions indicated by red arrows. B. Note the light brown compounds accumulated spread in the whole intercellular space, indicated by red asterisks in cross section whitouth reagent. C. Positive reaction for polysaccharides (PARS reaction) in the apoplast, indicated by red asterisks. D. Hypertrophy (Hp) of spongy parenchyma cells. A-C. *Miconia cabucu;* D. *Schinus terebinthifolia*. Bars= 25 µm.


A
B
C
D
Hp

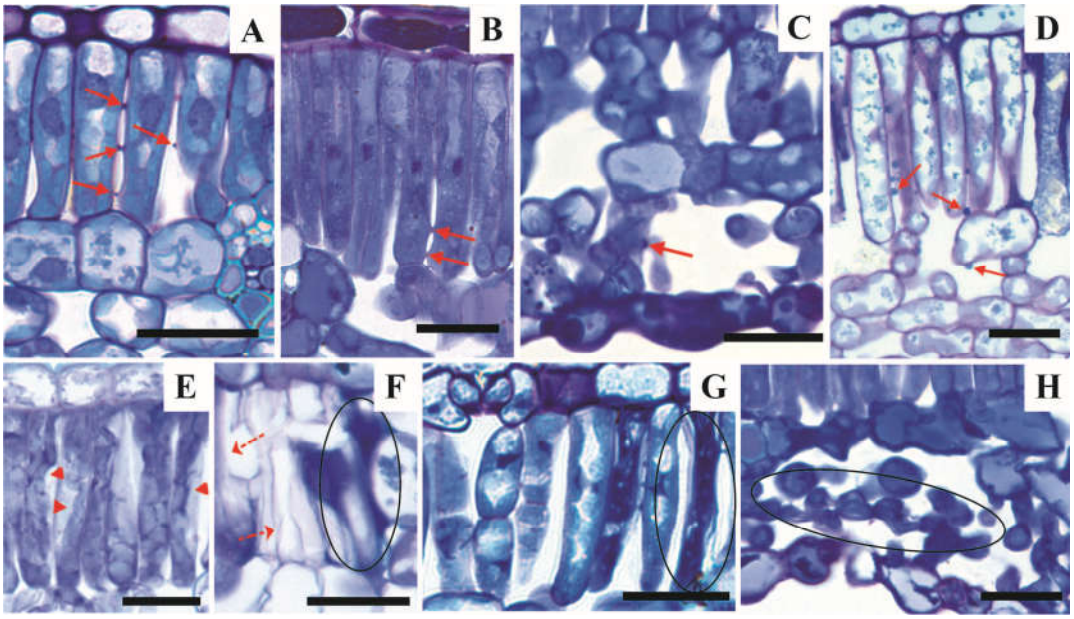



**Fig. 3.** Microscopic markers in mesophyll cells of potential intermidiate tolerant species to oxidative stress from different remnants of the Atlantic Forest. A-D. Wart-like protrusions of palisade parenchyma cells and spongy parenchyma cells indicated by red arrows. E. Protoplast changes indicated by red arrowheads. F. Parenchyma cells disruption (without content, red dotted arrows) and plasmolysed palisade parenchyma cells (ellipse), showing disorganized and condensed content. G-H. Plasmolysed (ellipse) palisade parenchyma cells showing disorganized and condensed content and spongy parenchyma cells. A. *Alchornea triplinervia*; B. *Piptadenia gonoacantha*; C. *Piptadenia gonoacantha;* D and F. *Solanum granulosoleprosum*; E and G. *Alchornea triplinervia*; H, *Piptadenia gonoacantha*. Bars= 25 µm.

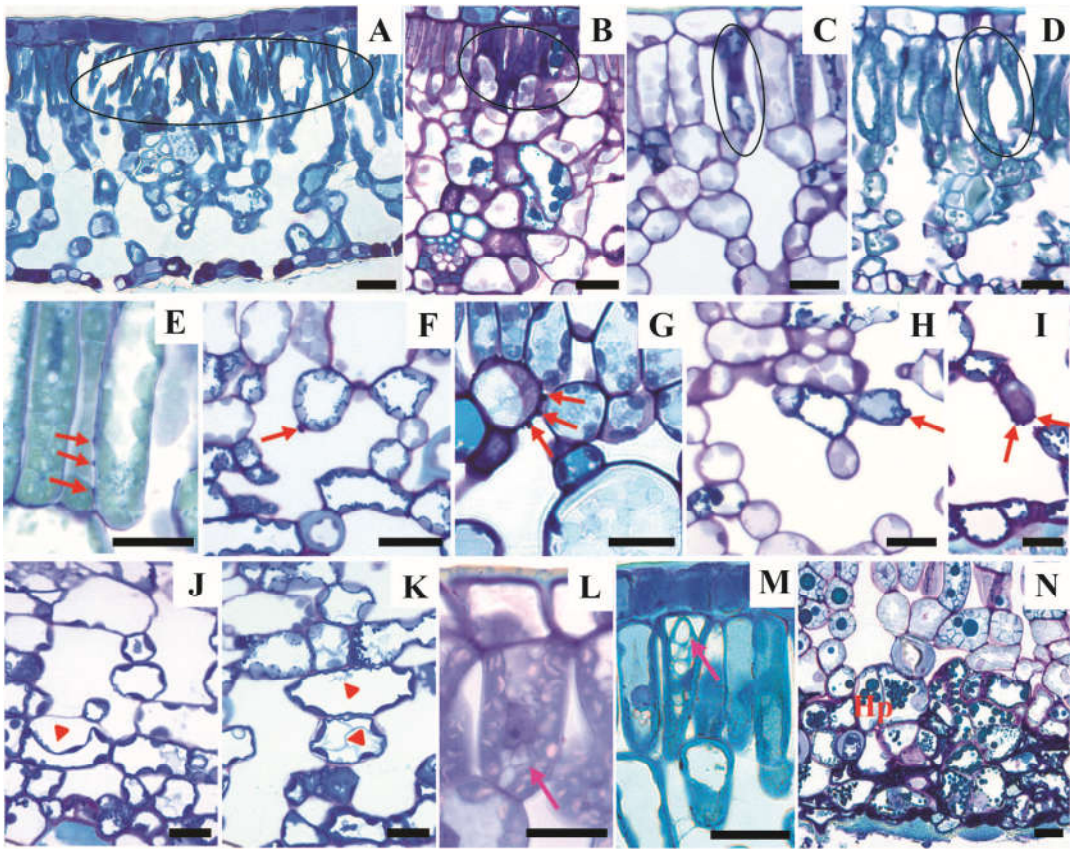



**Fig. 4.** Structural changes in mesophyll cells of potential sensitive species to oxidative stress from different remnants of the Atlantic Forest. A-D. Group of plasmolyzed (ellipse) palisade parenchyma cells. E-I. Wart-like protrusions of palisade cells and spongy parenchyma cells indicated by red arrows. F-I. Group of plasmolyzed (circle) palisade parenchyma cells. J-K. Protoplast changes indicated by red arrowheads. L-M. Vacuome (pink arrows) inside the palisade parenchyma cells. N. Hypertrophy (Hp) ofspongy parenchyma cells. A and I. *Ocotea paranapiacabensis;* B, J and N. *Drimys brasiliensis*; C and G. *Guarea kunthiana*; D, H and L. *Guarea macrophylla*; E and K. *Myrsine umbellate*; F and M. *Amaioua intermedia*. Bars= 25 µm.

**Table 4.** Frequency of microscopic markers (%) in mesophyll of tree group of species with distinct potential of tolerance to oxidative stress by environmental stressors sampled in different remnants of the Atlantic Forest during wet and dry period.

| Species | Potential of tolerance to oxidative stress | Wart-like protrusions | Plasmolysed cells | Protoplast changes | Vacuome | Hypertrophy | Compounds accumulated in the apoplast |
|---|---|---|---|---|---|---|---|
| **Wet period** | | | | | | | |
| | Tolerant | | | | | | |
| *M. cabucu* | | 47 | 0 | 0 | 0 | 0 | 35 |
| *S. terebinthifolius* | | 0 | 0 | 0 | 0 | 17 | 0 |
| | Intermidiate | | | | | | |
| *P. gonoacantha* | | 33 | 17 | 0 | 0 | 0 | 0 |
| *A. triplinervia* | | 33 | 50 | 50 | 0 | 0 | 0 |
| *S. granulosoleprosum* | | 17 | 17 | 0 | 0 | 0 | 0 |
| | Sensitive | | | | | | |
| *D. brasiliensis* | | 83 | 0 | 33 | 0 | 17 | 0 |
| *G. kunthiana* | | 33 | 17 | 0 | 0 | 0 | 0 |
| *G. macrophylla* | | 33 | 33 | 0 | 8 | 0 | 0 |
| *A. intermedia* | | 0 | 17 | 0 | 0 | 0 | 0 |
| *O. paranapiacabensis* | | 0 | 17 | 0 | 0 | 17 | 0 |
| *M. umbellata* | | 33 | 0 | 17 | 0 | 0 | 0 |
| **Dry period** | | | | | | | |
| | Tolerant | | | | | | |
| *M. cabucu* | | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. terebinthifolius* | | 0 | 0 | 0 | 0 | 0 | 0 |
| | Intermidiate | | | | | | |
| *P. gonoacantha* | | 17 | 17 | 0 | 0 | 0 | 0 |
| *A. triplinervia* | | 50 | 17 | 33 | 0 | 0 | 0 |
| *S. granulosoleprosum* | | 33 | 50 | 0 | 0 | 0 | 0 |
| | Sensitive | | | | | | |
| *D. brasiliensis* | | 0 | 17 | 17 | 0 | 0 | 0 |
| *G. kunthiana* | | 33 | 0 | 0 | 0 | 0 | 0 |
| *G. macrophylla* | | 50 | 17 | 17 | 0 | 0 | 0 |
| *A. intermedia* | | 0 | 0 | 17 | 17 | 0 | 0 |
| *O. paranapiacabensis* | | 17 | 17 | 0 | 0 | 0 | 0 |
| *M. umbellata* | | 17 | 33 | 0 | 0 | 0 | 0 |

## 4. Discussion

The methods designed to describe the leaf anatomy also provide tools to identify and quantify the structural microscopic markers typically associated with the impact of environmental stressors and help to understanding the response mechanisms of plants to oxidative stress (Kivimäenpää et al. 2003; Vollenweider et al. 2003; Pedroso et al. 2016). The wart-like protrusions are typical markers of oxidative burst by heavy metals (Hermle et al., 2007), ozone alone (Günthardt-Goerg et al., 1997; Vollenweider, 2003; Bussotti et al., 2005; Günthardt-Goerg and Vollenweider, 2007; Moura et al., 2018) or combined with $NO_2$ (Günthardt-Goerg et al., 1996) or drought (Pääkkonen et al., 1998). The protrusions may be composed by pectins (Günthardt-Goerg et al., 1997, 2000; Vollenweider, 2003, Bussotti et al., 2005; Hermle et al., 2007; Pedroso et al., 2016; Moura et al., 2018) and proteins (Günthardt-Goerg et al., 1996; 1997). Although unexpected, this marker was also observed in leaves of potential tolerant species sampled during the wet period. This finding may indicate a synergic effect of air pollutants and climatic conditions, such as high temperature and radiation during the wet period in the region of study. This assumption is reinforced by results obtained by Esposito et al. (2018), who evaluated the oxidant-antioxidant balance in the same tree species included in the present study. The authors observed that air temperature, global solar radiation, ozone and nitrogen dioxide promoted increases in the leaf contents of ROS and derivatives of lipid peroxidation in pioneer and non-pioneer trees during the wet period.

In addition, the widespread microscopic changes observed in all species may simply reflect the better environmental conditions for maximized photosynthesis during the wet period in SE Brazil, which coincides with spring and summer times. As discussed by Aguiar-Silva et al. (2016), who analyzed the antioxidant responses of tree

species sampled in the ecological area of relevant interest Mata de Santa Genebra (MSG), the wet season in that region is favorable for plant growth, due to a higher availability of light energy, water, and optimum temperatures for photosynthesis, which in turn intensify the ROS formation in plant cells, thus increasing the photo-oxidative stress. The higher incidence of solar radiation during this period may increase the leaf temperature, which can also increase the oxidative stress (Bussotti, 2008). In contrast, tolerant species seem to have intrinsic functional leaf traits (including biochemical and/or morpho-anatomical traits; according to results presented in chapter 1 and in Brandão et al., 2017 and Esposito et al., 2018) that confer them a higher ability to acclimate to the environmental conditions in dry period (e.g. high concentrations of gaseous pollutants observed next to the forest remnants studied; Brandão et al., 2017; Esposito et al., 2018) than the intermediate and sensitive species, which explains the absence of wart-like protrusions in the tolerant species in this period.

The hypertrophy (i.e. increase in cell size) of the spongy parenchyma cells occurred in leaves of potential tolerant and sensitive species sampled during wet period. Fernandes et al. (2019) suggesting that the hypertrophy is a response that increasing the resistance to gaseous pollution diffusion. This response may also indicate acclimation to synergic effects of air pollutants and climatic conditions during the wet period in the region of study. In other words, the common features among groups (notably wart-like protrusions and hypertrophy) indicates that plants adopt the same strategies to deal with oxidative pressure existing in their natural environment, especially in wet period.

In contrast, the plasmolysed parenchyma cells, invaginated plasma membrane inside the protoplasts, vacuome and compounds accumulated in the apoplast represent species-specific microscopic markers, which differentiate the plant species regarding to their capacity to act against oxidative stress.

The plasmolysed parenchyma cells, invaginated plasma membrane of parenchyma cells and vacuome were characteristic of species with some degree of sensitivity to oxidative stress (intermediate/sensitive-specifc responses). Partial plasmolysis of cells and invaginated plasma membrane can be consequences of alterations in the permeability of cell membranes and may result in a sudden loss of membrane integrity, culminating in totally plasmolysed cells (Günthardt- Goerg and Vollenweider, 2007). In general, the plamolysed cells showed condensation of their content, and the accumulation of antioxidants with dense appearance, characteristic of programmed cell death (PCD) (Fernandes et al., 2016). The structural membrane changes were induced by lipid oxidation and proteins oxidation, the last more sensitive to oxidative stress according to Roschina and Roschina (2003). These biochemical alterations are commonly found in sensitive plants exposed to oxidative stressors and without effective enhancement antioxidant system (Aguiar-Silva et al., 2016).

We identified that the compounds in the apoplast are oxidized polyphenols, which are responsible for the brownish coloration on tissues (Fernandes et al., 2016). Constitutively, glycosylated derivatives of flavonoids are found in the apoplast (Agati et al., 2012; Brooker et al., 2012) and they are the least toxic form for plants. However, their concentrations may be altered in the presence of atmospheric pollutants (Booker et al., 2012). The same microscope marker was described in seedlings of *Astronium graveolens* (a native tree species from de Altantic forest) exposed in the field with high concentrations of $O_3$ (Fernandes, 2015; Fernandes et al., 2016). The phenolic glycosides are effective reactive oxygen scavengers (Agati et al., 2012; Booker et al., 2012; Fernandes et al., 2016) and can play an important strategy for mitigating the oxidative pressure in the group of tolerant species, Therefore, the accumulation of phenolic glycosides observed seems to be a new microscopic marker in tolerant species, to

oxidative stress in tropical environments.

Finally, we may recommend further experimental studies in order to confirm the new scientific contribution obtained in the present field study and to determine the most adequate microscopic markers for indicating the potential tolerance of tropical plants to oxidative stress induced by synergic effects of multiple stress factors.

## 5. Conclusions

Microscopic markers were observed in leaf blades of tropical trees without visible injuries. Their potential tolerance level to oxidative stress previously defined by morpho-anatomical traits was validated specific microscopic markers.

Tree species with some degree of sensitivity to environmental stress (intermediate tolerant and sensitive species) showed greater variety of microscopic markers and higher frequency of oxidative markers (wart-like protrusions, plasmolyzed cells, protoplast changes and vacuome), which confirm our hypothesis.

Potential tolerant species presented a lower variety and frequency of microscopic markers. In addition, these species showed microscopic markers that are indicators of increased resistance against oxidative stress, such as hypertrophy of mesophyll cells and accumulation of phenolic glycosides in the apoplast that increase the cellular defense against reactive oxygen species.

We showed that certain microscopic markers, such as wart-like protrusion and hypertrophy that were widely observed in tree species with distinct tolerance levels, can reflect the natural climatic seasonality in the studied region.

Finally, we concluded that accumulation of phenolic glycosides in apoplast is a specific microscopic marker identified in leaves of tropical tolerant species, which differentiates them from the other plant species regarding to the capacity to handle the

oxidative stress.

**Acknowledgments**

The authors gratefully acknowledge the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the PhD granted to the first author; Municipal Park Paranapiacaba, State Park Fontes do Ipiranga, Ecological Area of Relevant Interest Mata de Santa Genebra and State Park Itacolomi for permitting the leaf collections; Dr. Eduardo Pereira Cabral Gomes, Dr. Hildeberto Caldas de Sousa and Dr. Maria Cristina Teixeira Braga for helping in tree species selection; Amariles C. de Souza, Douglas D. Santos, Giovanna Boccuzzi, Marisia P. Esposito, Marcela R.G.S. Engela, Ricardo K. Nakazato and Tiago A. Tassinari for collection of plant material during field work.

## 6. References

Agati, G., Azzarello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: location and functional significance. Plant Sci. 196, 67–76.

Aguiar-Silva, C., Brandão, S.E., Domingos, M., Bulbovas, P., 2016. Antioxidant responses of Atlantic Forest native tree species as indicators of increasing tolerance to oxidative stress when they are exposed to air pollutants and seasonal tropical climate. Ecol Indic. 63, 154–164.

Alves, E.S., Moura, B.B., Pedroso, A.N.V., Tresmondi, F., Machado, S.R., 2016. Cellular markers indicative of ozone stress on bioindicator plants growing in a tropical environment. Ecol Indic. 67, 417–424.

André, O., Vollenweider, P., Günthardt-Goerg, M.S., 2006. Foliage response to heavy metal contamination in Sycamore Maple (*Acer pseudoplatanus* L.) For. Snow Landsc Res. 80, 275–288.

Brandão, S.E., Bulbovas, P., Lima, M.E., Domingos, M., 2017. Biochemical leaf traits as indicators of tolerance potencial in tree species from the Brazilian Atlantic Forest against oxidative environmental stressors. Sci Total Environ. 575, 406–417.

Booker, F.L., Burkey, K.O., Jones, A.M., 2013. Re-evaluating the role of ascorbic acid and phenolic glycosides in ozone scavenging in the leaf apoplast of *Arabidopsis thaliana* L. Plant Cell Environ. 35, 1456–1466.

Bussotti, F., 2008. Functional leaf traits, plant communities and acclimation processes in relation to oxidative stress in trees: a critical overview. Glob Chang Biol. 14, 2727–2739.

Bussotti, F., Agati, G., Desotgiu, R., Matteini, P., Tani, C., 2005. Ozone foliar symptoms in woody plant species assessed with ultrastructural and fluorescence analysis. New Phytol. 166, 941–955.

Calatayud, V., García-Breijo, F.J., Cervero, J., Reig-Arminãn, J., Sanz, M.J., 2011 Physiological, anatomical and biomass partitioning responses to ozone in the Mediterranean endemic plant Lamottea dianae. Ecotox Environ Safe. 74, 1131–1138.

Cardoso-Gustavson, P., Fernandes, F.F., Alves, E.S., Pereire, M.V., Moura, B.B., Domingos, M., Rodrigues, C.A., Ribeiro, A.P., Nievola, C.C., Figueiredo, A.M.G., 2015. *Tillandsia usneoides*: a successful alternative for biomonitoring changes in air quality due to a new highway in São Paulo, Brazil. Environ Sci and Pollut R. 23, 1779–1788.

Domingos, M., Bulbovas, P., Camargo, C.Z.S., Aguiar-Silva, C., Brandão, S.E., Dafré-Martinelli, M., Dias, A.P.L., Engela, M.R.G.S., Gagliano, J., Moura, B.B., Alves, E.S., Rinaldi, M.C.S., Gomes, E.P.C., Furlan, C.M., Figueiredo, A.M.G., 2015. Searching for native tree species and respective potential biomarkers for future assessment of pollution effects on the highly diverse Atlantic Forest in SE-Brazil. Environ Pollut. 202, 85–95.

Esposito, M.P., Nakazatu, R.K., Pedroso, A.V.P., Lima, M.E.L., Figueiredo, M.A., Diniz, A.P., Kozovits, A.R., Domingos, M., 2018. Oxidant-antioxidant balance and tolerance against oxidative stress in pioneer and non-pioneer tree species from the remaining Atlantic Forest. Sci Total Environ. 625, 382–393.

Feder, N., O'Brien., 1968. Plant microtechnique: some principles and new methods. Am J Bot. 55, 123–142.

Fernandes, F.F. 2015. Marcadores microscópicospara a validação de sintomasem espécie nativa a ser empregada no biomonitoramento de ozônio (M.Sc. thesis). Insituto de Botânica, São Paulo.

Fernandes, F.F., Cardoso-Gustavson, P., Alves, E.S., 2016. Synergism between ozone and light stress: structural responses of polyphenols in a woody Brazilian species. Chemosphere 155, 573–582.

Fernandes, F.F., Esposito, M.P., Engela, M.R.G. da S., Cardoso-Gustavson, P., Furlan, C.M., Hoshika, Y., Carrari, E., Magni, G., Domingos, M., Paoletti, E., 2019. The passion fruit liana (*Passiflora edulis* Sims, Passifloraceae) is tolerant to ozone. Sci Total Environ. 656, 1091–1101.

Fink, S., 1999. Pathological and regenerative plant anatomy. Encyclopedia of Plant Anatomy. vol. XIV/6. Gebrüder Bornträger, Berlin, Stuttgart. pp. 523–527.

Gahan, P.B., 1984. Plant histochemistry and cytochemistry. London: Academic Press.

Gerlach, D., 1984. Botanische Mikrotechnik. 3. Auflage. Stuttgart: Thieme Verlag.

Gravano, E., Bussotti, F., Strasser, R.J., Schaub, M., Novak, K., Skelly, J., Tani, C., 2004. Ozone symptoms in leaves of woody plants in open-top chambers: ultrastructural and physiological characteristics. Physiol Plant. 121, 620–633.

Guerrero, C.C., Günthardt-Goerg, M.S., Vollenweider, P., 2013. Foliar Symptoms Triggered by Ozone Stress in Irrigated Holm Oaks from the City of Madrid, Spain PLoS ONE 8:e69171

Günthardt-Goerg, M.S., McQuattie, C.J., Maurer, S., Frey, B., 2000. Visible and microscopic injury in leaves of five deciduous tree species related to current critical ozone levels. Environ Pollut. 109, 489–500.

Günthardt-Goerg, M.S., McQuattie, C.J., Scheidegger, C., Rhiner, C., Matyssek, R., 1997. Ozone-induced cytochemical and ultrastructural changes in leaf mesophyll cell. Can J of Forest Res. 27, 453–463.

Günthardt-Goerg, M.S., Vollenweider, P., 2007. Linking stress with macroscopic and microscopic leaf response in trees: new diagnostic perspectives. Environmental Pollution 147, 467–488.

Hermle, S., Vollenweider, P., Gunthardt-Goerg, M. S., McQuattie, C. J., Matyssek, R., 2007. Leaf responsiveness of *Populus tremula* and *Salix viminalis* to soil contaminated with heavy metals and acidic rainwater. Tree Physiol. 27, 1517–1531.

Kivimäenpää, M., Jonsson, A.M., Stjernquist, I., Sellden, G., Sutinen, S. 2004. The use of light and electron microscopy to assess the impact of ozone on Norway spruce needles. Environ Pollut. 127, 441–453.

Kivimäenpää, M., Riikonen, J., Sutinen, S., Holopainen, T. 2014. Cell structural changes in the mesophyll of Norway spruce needles by elevated ozone and elevated temperature in open-field exposure during cold acclimation. Tree Physiol. 34, 389–403.

Kivimäenpää, M., Sutinen, S., Karlsson, P.E., Sellden, G. 2003. Cell structural changes in the needles of Norway spruce exposed to long-term ozone and drought. Annals of Botany. 92, 779–793.

Moura, B.B., Alves, E.S,Marabesi, M.A, Souza, S.R, Schaub, M., Vollenweider, P., 2018. Ozone affects leaf physiology and causes injury to foliage of native tree species from the tropical Atlantic forest of southern Brazil. Sci Total Environ. 610-611, 912–925.

Moura, B.B., Alves, E.S., Marabesi, M.A., de Souza, S.R., Schaub, M., Vollenweider, P. 2018. Ozone affects leaf physiology and causes injury to foliage of native tree species from the tropical Atlantic Forest of southern Brazil. Sci Total Environ 610–611, 912–925.

Moura, B.B., Alves, E.S., Souza, S.R., Domingos, M., Vollenweider, P. 2014. Ozone phytotoxic potential with regard to fragments of the Atlantic semi-deciduous Forest downwind of Sao Paulo, Brazil. Environ Pollut, 192, 65–73.

Moura, B.B., Souza, S.R., Alves, E.S., 2011. Structural responses of *Ipomoea nil* (L.) Roth “Scarlet O'Hara” (Convolvulaceae) exposed to ozone. Acta Bot Bras. 25, 122–129.

Munch, L. 1989. Fluorescence Analysis in Food. Longman Scientific and Technical, Harlow.

Nakazato, R.K., Esposito, M.P., Cardoso-Gustavson, P., Bulbovas, P., Pedroso, A.N.V., Assis, P.I.L., Domingos, M. 2018. Efficiency of biomonitoring methods applying tropical bioindicator plants for assessing the phytoxicity of the air pollutants in SE, Brazil. Sci Total Environ. 25,19323–19337.

Orendovici, T., Skelly, J.M., Ferdinand, J.A., Savage, J.E., Sanz, M.J., Smith, G.C. 2003. Response of native plants of northeastern United States and southern Spain

to ozone exposures; determining exposure/response relationships. Environ Pollut. 125, 31–40.

Pääkkönen, E., Günthardt-Goerg, M.S., Holopainen, T., 1998. Responses of leaf processes in a sensitive birch (*Betula pendula* Roth) clone to ozone combined with drought. Ann Bot. 82, 49–59.

Paoletti, E., Contran, N., Bernasconi, P., Günthardt-Goerg, M.S., Vollenweider, P., 2009. Structural and physiological responses to ozone in Manna ash (*Fraxinus ornus L.*) leaves of seedlings and mature trees under controlled and ambient conditions. Sci Total Environ. 407, 1631–1643.

Pedroso, A.N.V., Alves, E.S. 2015. Temporal dynamics of the cellular events in tobacco leaves exposed in São Paulo, Brazil, indicate oxidative stress by ozone. Environ. Sci Pollut Res Int. 22, 6535–6545.

Pedroso, A.N.V., Bussotti, F., Papini, A., Tani, C., Domingos, M., 2016. Pollution emissions from a petrochemical complex and other environmental stressors induce structural and ultrastructural damage in leaves of a biosensor tree species from the Atlantic Rain Forest. Ecol Indic. 67, 215–226.

Sant'Anna-Santos, B.F., Silva, L.C., Azevedo, A.A., Aguiar, R. 2006. Effects of simulated acid raind on leaf anatomy and micromorphology of Genipa americana L. (Rubiaceae). Braz Arch Biol Technol. 49, 313–332.

Schaub, M., Calatayud, V., Ferretti, M., Brunialti, G., Lövblad, G., Krause, G., Sanz, M.J. 2016. Part VIII: Monitoring of Ozone Injury. In: UNECE ICP Forests Programme Co-ordinating Centre (ed.): Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. Thünen Institute of Forest Ecosystems, Eberswalde, Germany, 14 p. Annex [http://www.icp-forests.org/manual.htm].

Silva, L.D., Oliva, M.A., Azevedo, A.A., Araújo, J.M., Aguiar, R.M., 2005 Micromorphological and anatomical alterations caused by simulated acid rain in Restinga plants: *Eugenia uniflora* and *Clusia hilariana*. Water Air Soil Pollut . 168, 129–143.

Tresmondi, F., Alves, E.S., 2011 Structural changes in *Psidium guajava* 'Paluma'leaves exposed to tropospheric ozone. Acta Bot. Bras. 25, 122–129.

Vollenweider, P., Cosio, C., Gunthardt-Goerg, M.S., Keller, C. 2016. Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.) Part II Microlocalization and cellular effects of cádmium. Environ Exper Bot. 58, 25–40.

Vollenweider, P., Fenn, M.E., Menard, T., Günthardt-Goerg, M., Bytnerowicz, A. 2013. Structural injury underlying mottling in ponderosa pine needles exposed to ambient ozone concentrations in the San Bernardino Mountains near Los Angeles, California. Trees. 27, 895- 911.

Vollenweider, P., Ottiger, M., Günthardt-Goerg, M.S., 2003. Validation of leaf ozone symptoms in natural vegetation using microscopical methods. Environ Pollut 124, 101– 118.

Roschina, V.V., Roschina, V.D. 2003. Ozone and plant cell. Dordrecht, Kluwer Academic Publishers.

Capítulo 3

## The passion fruit liana (*Passiflora edulis* Sims, Passifloraceae) is tolerant to ozone[7]

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[7] Artigo publicado na Science of the Total Environment (DOI:https://doi.org/10.1016/j.scitotenv.2018.11.425).

Science of the Total Environment 656 (2019) 1091–1101

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# The passion fruit liana (*Passiflora edulis* Sims, Passifloraceae) is tolerant to ozone

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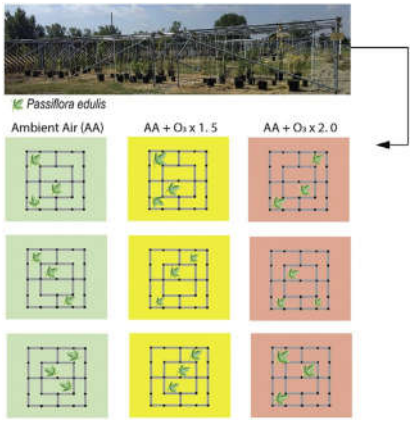
## HIGHLIGHTS

- Seedlings of *Passiflora edulis* were exposed to ozone in a FACE system.
- Anatomical changes observed in leaf tissue may restrain damage progression.
- A high level of $O_3$ did not affect physiological processes.
- Biochemical leaf traits enable *P. edulis* to tolerate oxidative stress.
- *P. edulis* can be considered an $O_3$-tolerant species.

## GRAPHICAL ABSTRACT




## article info

*Article history:*
Received 1 October 2018
Received in revised form 23 November 2018
Accepted 28 November 2018
Available online 30 November 2018

Editor: Pavlos Kassomenos

*Keywords:*
Anatomical acclimatization
Antioxidant system
Liana
Oxidative stress
Polyphenols

## abstract

*Passiflora edulis* Sims is a liana species of high economic interest and is an interesting model plant for understanding ozone action on disturbed vegetation. In this work we hypothesized that *P. edulis* has adaptive responses to oxidative stress that enable it to tolerate ozone damage based on its capacity to grow under a diversity of environmental conditions and to dominate disturbed areas. We exposed seedlings to three levels of ozone in a Free-Air Controlled Exposure (FACE) system (22, 41 and 58 ppb h AOT40 and 13.52, 17.24 and 20.62 mmol $m^{-2}$ PODo, over 97 days) for identifying its tolerance mechanisms. Anatomical (leaf blade structure and fluorescence emission of chloroplast metabolites), physiological (leaf gas exchange, growth rate and biomass production) and biochemical (pigments, total sugars, starch, enzymatic and non-enzymatic antioxidant metabolites, reactive oxygen species and lipid peroxidation derivatives) responses were assessed. Ozone caused decreased total number of leaves, hyperplasia and hypertrophy of the mesophyll cells, and accelerated leaf senescence. However, $O_3$ did not affect carbohydrates content, net photosynthetic rate, or total biomass production, indicating that the carboxylation efficiency and associated physiological processes were not affected. In addition, *P. edulis* showed higher leaf contents of ascorbic acid, glutathione (as well high ratio between their reduced and total forms), carotenoids, and flavonoids located in the chloroplast outer envelope membrane.

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Tropical environment
Vine species

Our results indicate that *P. edulis* is an $O_3$-tolerant species due to morphological acclimation responses and an effective antioxidant defense system represented by non-enzymatic antioxidants, which maintained the cellular redox balance under ozone.



## 1. Introduction

Lianas – climbing vines with woody stems – are a key component of most tropical forests due to their abundance, constituting 10% to 45% of the woody species (Pivello et al., 2018). They are also the second life-form in biomass production after trees (DeWalt and Chave, 2004; Pérez-Salicrup et al., 2004). They ascend in suitable hosts, and live for decades, occurring also in mid-to-late successional forests (Rossell and Eggleston, 2017). When present in a high density, they may further reduce tree growth and alter tree species composition, changing the forest physiognomy and reducing the capacity of forests to sequester atmospheric carbon (Phillips et al., 2002; Schnitzer and Bongers, 2002; Pivello et al., 2018). It is thus important to predict the reasons of the high abundance of lianas in tropical forests (Schnitzer, 2005; Pivello et al., 2018). The high capacity of hydraulic redistribution and water storage, multifocal growth, drought resilience and acquisitive resource syndrome are among them (Amorim et al., 2018).

*Passiflora* is the largest genus of Passifloraceae, comprising about 520 species (Wohlmuth et al., 2010) predominantly found in tropical and subtropical regions (Dhawan et al., 2004; Araújo et al., 2017). Its distribution is directly determined by the environmental conditions, such as seasonality in temperature and rain precipitation (Scherer, 2014). Brazil is the hotspot in Passifloraceae biodiversity, including *Passiflora edulis* Sims, which is distributed in heterogeneous environments and in different phytogeographic domains (Scherer, 2014; Flora do Brasil 2020, under construction). In addition, *P. edulis* shows a higher ability to adapt and acclimate to drought than other *Passiflora* species, as reported in a controlled experiment (Souza et al., 2018).

Tropospheric ozone ($O_3$) is a strong oxidizing air pollutant formed through a complex series of reactions involving the action of sunlight on nitrogen dioxide and hydrocarbons (Ashmore, 2005; Lodovici and Bigagli, 2011). The tolerance level of plants to $O_3$ depends on their absorption rate, constitutive detoxification capacity (enzymatic and non-enzymatic antioxidant metabolites), carboxylase ratio and capacity of antioxidant regeneration (Matyssek et al., 2012). After entrance, $O_3$ reacts with water, leading to the formation of reactive oxygen species (ROS) at the interface of cell wall. The oxidative destruction of lipids and proteins of the plasma membrane and production of other free radicals and reactive intermediates, is a process known as lipid peroxidation (Kanofsky and Sima, 2005; Puckette et al., 2007). Since the half-lives of mostly ROS are extremely short, stable end products of oxidative damage in cell macromolecules are used to measure the oxidative stress (Bhaduri and Fulekar, 2012). ROS may cause oxidative lesions that result in anatomical leaf changes (Vollenweider et al., 2003), and in negative impacts on plant metabolism and photosynthesis that may progress to the appearance of visible leaf $O_3$ injury and reduction of plant growth and development (Hernandez-Gimenez et al., 2002; Matyssek and Sandermann, 2003).

We assumed that *P. edulis* is an interesting model species for understanding $O_3$ action on disturbed vegetation, given its wide distribution in the tropics (Flora do Brasil 2020, under construction) and its high abundance in disturbed vegetation by human action, where high $O_3$ levels are expected. Considering its vine characteristics above mentioned that enable it to tolerate natural environmental stresses, we raised the hypothesis that *P. edulis* also has adaptive responses that enable it to tolerate the oxidative stress caused by $O_3$. The knowledge on the tolerance level of *P. edulis* to $O_3$ is also of economic interest because several *Passiflora* species are widely cultivated in tropical and subtropical regions. The fruits of *P. edulis* are, in particular, the most consumed among several species of *Passiflora* in the international market (Ocampo et al., 2010; Souza et al., 2018). In addition, *Passiflora* species have great pharmacological value (Dornelas et al., 2006) because of the bioactive compounds found in the aerial vegetative organs (Otobone et al., 2005; Yudasheva et al., 2005; Castro et al., 2007).

Aiming to test the above mentioned hypothesis, we assessed anatomical (leaf blade structure and fluorescence emission of chloroplast metabolites), physiological, (leaf gas exchange, stomatal ozone uptake, growth rate, biomass production) and biochemical (pigments, primary and secondary metabolites, antioxidants, reactive oxygen species and lipid peroxidation derivatives) responses in plants exposed to ozone in a Free-Air Controlled Exposure (FACE) system.

## 2. Materials and Thethods

### 2.1. Experimental design

Seedlings of *P. edulis* (approx. 20 cm high) were obtained from an Italian nursery (43.935351 N, 10.928174 E) and transplanted to 17 L pots filled with a mixture of sand: peat: nursery soil (1:1:1, v/v). Plants were irrigated every afternoon by a drip irrigation system to avoid water stress (i.e., volumetric soil water content was maintained to the field capacity of ≈0.295 $m^3$ $m^{-3}$, Paoletti et al., 2017), and fertilized with N:P:K (10:10:10) every 7 days during the first and second month of exposure, and once in the last month of the experiment.

The experiment was carried out in an $O_3$ FACE system located in Sesto Fiorentino, Florence, Italy (43°48′59″ N, 11°12′01″ E, 55 m a.s.l.). Details of this experimental facility are given in Paoletti et al. (2017). The plants were submitted to three $O_3$ levels: ambient air (AA); intermediate ozone level (AA + $O_3$ × 1.5) and elevated ozone level (AA + $O_3$ × 2.0) during 97 days of summer season (from June 10th to September 15th, 2017). The system consisted of three plots per $O_3$ treatment; each plot (5 × 5 × 2 m, L × W × H respectively) was considered as a replicate. Three pots of *P. edulis* were maintained in each plot (n = 3, totalizing nine plants per $O_3$ treatment = 27 plants).

The $O_3$ concentration was continuously monitored in one plot per $O_3$ treatment using monitors (Mod. 202, 2B Technologies, Boulder CO, USA) and the AOT40 (accumulated $O_3$ exposure over a threshold of 40 ppb) was calculated during daylight hours (global radiation N 50 W $m^{-2}$), following the protocol described in CLRTAP (2017). Global solar radiation (GSR), temperature (Temp), relative humidity (RH) and precipitation (P) were continuously recorded during the experiment by a Watchdog station (Mod. 2000; Spectrum Technology, Inc., Aurora, IL, USA) at 2.5 m a.g.l.

### 2.2. Anatomical responses

Samples for microscopic evaluation were gathered on leaves collected at the end of the experiment. We sampled symptomatic leaves (chlorosis induced by $O_3$, as shown in Fig. S1 of the Supplementary material) of plants from all treatments and asymptomatic leaves only of plants from the AA treatment. Fragments (approx. 1 $cm^2$) of the median region of both asymptomatic and symptomatic leaves were fixed in 2.5% glutaraldehyde buffered at pH 7.0 with 0.067 M Sorensen phosphate buffer, and placed under vacuum before storing at 4 °C.

Part of the fragments destined to confocal analyses (Zeiss LSM 510-Meta) were washed in distilled water, cut at 20 μm thickness by a cryomicrotome (Leica CM1100), and mounted in Fluoromount (Sigma-Aldrich) aqueous medium. The samples were excited with a

364 nm laser to promote polyphenol (Fernandes et al., 2016) and carotenoid emissions (Roshchina, 2008; D'Andrea et al., 2014). Emissions were taken from 400 to 607 nm and subsequently from 400 to 700 nm in a lambda stack mode (a series of images from the same microscopic region with different wavelengths with 10 to 11 nm increments) for better visualization of chloroplast metabolites. In each image (objective lens 40×), six chloroplasts of the palisade parenchyma were randomly selected and the intensities of metabolite emission in the obtained spectra (400–607 nm and 400–700 nm) were quantified using the software Zeiss LSM Image Browser. The emission wavelength (nm) between 450 and 500 nm referred to phenolic compounds, 500–550 nm to carotenoids, and above 650 nm to chlorophylls (chl *a* and *b*).

The other part of the fragments was dehydrated in an ethanol series, embedded in Technovit 7100 historesin and transversally sectioned to 1.5 μm semi-thin cuttings by a rotary microtome Leica (RM2245) for structural and histochemical analyses. Material was stained with toluidine blue and *p*-phenylenediamine for metachromasy and lipid identification, respectively (Feder and O'Brien, 1968; Kivimäenpää et al., 2004), PAS reaction for polysaccharides (Gahan, 1984) and Comassie blue for proteins (Wetzel et al., 1989).

### 2.3. Physiological responses

#### 2.3.1. Leaf gas exchange and stomatal ozone uptake (POD)

Gas exchange measurements of fully expanded sun leaves (4th to 6th from the shoot tip) were carried out by a portable infrared gas analyzer (CIRAS-2 PP Systems, Herts, UK). On 11–14th September, the measurements were taken with a control value of photosynthetic photon flux density (PPFD) of 1500 μmol $m^{-2} s^{-1}$, ambient $CO_2$ concentration (Ca) of 400 μmol $mol^{-1}$, relative humidity of 40 to 60% and leaf temperature of 25 °C, from 9:00 to 12:00 h. We determined the light-saturated net photosynthetic rate ($A_{sat}$), stomatal conductance for water vapour ($g_{sw}$) and intercellular $CO_2$ concentration (Ci) at ambient $CO_2$ concentration (400 ppm) for calculating the Ci/Caratio.

The $O_3$ dose during the experiment was calculated as phytotoxic ozone dose ($POD_Y$) above an hourly stomatal uptake threshold of 0 nmol $m^{-2} s^{-1}$ ($POD_0$). $POD_Y$ is given as follows:

$$POD_Y = \sum \max(F_{st} - Y, \quad 0) \tag{1}$$

where $F_{st}$ is an hourly mean stomatal $O_3$ uptake (nmol $m^{-2} s^{-1}$) and Y is a species-specific threshold of stomatal $O_3$ uptake (nmol $m^{-2} s^{-1}$). As it was not clear which threshold Y can be applied to this species, we did not set a threshold i.e. Y = 0 ($POD_0$) in the present study. $F_{st}$ was calculated according to CLRTAP (2017), as follows:

$$F_{st} = [O_3] \cdot g_s \cdot \frac{r_c}{r_b + r_c} \tag{2}$$

where $[O_3]$ is the hourly $O_3$ concentration (ppb), $g_s$ is the stomatal conductance for $O_3$ (m $s^{-1}$), $r_c$ is the leaf surface resistance ($r_c = 1 / (g_s + g_{ext})$, where $g_{ext} = 0.0004$ m $s^{-1}$ indicates a cuticular and/or external leaf conductance), and $r_b$ is the leaf boundary layer resistance (m $s^{-1}$). Stomatal conductance was estimated by the multiplicative empirical model (Jarvis, 1976; CLRTAP, 2017; Hoshika et al., 2018). The detail is described in the Supplementary file.

#### 2.3.2. Growth rate and biomass production

Growth characteristics were monthly assessed by measuring plant height, stem diameter (near the base) and total number of leaves. The measuring times were referred as To (initial measurement) to T3 (final measurement). For the determination of stem height and diameter, a measuring tape and a digital caliper (data expressed in cm) (Digimess, São Paulo, Brazil) were used (Sá et al., 2014), respectively. Relative growth rate (RGR) was calculated every month in relation to the previous measurement (T1 − To; T2 − T1 and T3 − T2) and also between the initial and final measurements (T3 − To), using the formula proposed by Benincasa (1988).

At the end of the exposure period, leaves, stems/branches and roots of each plant were harvested, stored in paper packaging and dried in an oven at 60 °C until constant weight. The leaf, stem/branch and root biomasses of an additional lot of 5 plants were determined at the beginning of the experiment in order to obtain the To values. Shoot to root ratios were calculated according to Moura et al. (2017).

### 2.4. Biochemical responses

The biochemical responses were measured on a composite sample of four fully-expanded and sun-exposed leaves per plant, according to the methods described below. The composite leaf samples were stored in an ultra-freezer, under −80 °C. For biochemical responses, three analytical replicates were performed in asymptomatic leaf samples.

#### 2.4.1. Pigments

The pigment Chl *a* and *b* and carotenoids (CAR) content was determined in the same leaf extracts by spectrophotometric UV–vis method. The extracts were obtained by homogenizing frozen leaves in ethanol (96%). The supernatant was measured at 470 nm to determine the levels of carotenoids, at 649 nm to determine Chl *a* and at 666 nm to determine Chl *b* (Wintermans and De Mots, 1965).

#### 2.4.2. Primary and secondary metabolites

Total carbohydrate contents in the frozen leaf samples (100 mg) were extracted in 80% ethanol and determined colorimetrically at 490 nm using the phenol–sulfuric acid method (Dubois et al., 1956). Starch content was determined by using 10 mg of the freeze-dried residue after ethanol extraction. The absorbance was measured in an Elisa plate at 490 nm (Amaral et al., 2007).

The total flavonoids were extracted from the frozen leaves (100 mg) with 80% methanol in dry bath (at 70 °C for 1 h). The amounts of total flavonoids were quantified using aluminum chloride 5% method at 420 nm (Santos and Furlan, 2013).

#### 2.4.3. Antioxidants

Ascorbic acid, in its reduced (AsA) and total (totAA) forms, was analyzed in frozen leaves using the chromatographic method described by López et al. (2005) and a HPLC (Metrohm) connected to an UV–Vis detector.

Glutathione in its reduced (GSH), oxidized (GSSG) and total (totG) content was determined in frozen leaves according to the method described by Israr et al. (2006).

Superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities were analyzed by spectrophotometric UV–vis using extracts of frozen leaves. SOD and APX activities were determined according to a slightly modified version of the method described by Reddy et al. (2004). CAT activity was determined as described by Kraus et al. (1995) with some modifications proposed by Azevedo et al. (1998). The activity of glutathione reductase (GR) was determined in frozen leaves according to the method of Reddy et al. (2004).

Further analytical details about enzymatic and non-enzymatic compounds can be found in Esposito et al. (2016).

#### 2.4.4. Reactive oxygen species, malondialdehyde and hydroperoxide conjugated diene

The principle of the •OH radical assay was the quantification of the 2-deoxyribose degradation product, malondialdehyde (MDA), by its condensation with thiobarbituric acid (TBA). The reactions started by the addition of Fe (II) to solutions containing 2-deoxyribose, iron chelator, phosphate buffer (pH = 7.2) and then were stopped by the addition of phosphoric acid followed by TBA. The absorbance of this mixture was measured at 532 nm (Lopes et al., 1999).

The $H_2O_2$ contents were determined following Alexieva et al. (2001). The reaction mixture consisted of supernatant extract (frozen leaves + trichloroacetic acid), potassium phosphate buffer (100 mM, pH 7.0) and reagent potassium iodide (KI). The reaction was developed for 1 h in darkness and absorbance was measured at 390 nm.

The $O_2^{\bullet-}$ production rate was determined using the hydroxylamine oxidation method (Wang and Luo, 1990) with some modifications. The supernatant was mixed with potassium phosphate buffer (pH 7.8) and hydroxylamine chloride. *p*-Aminobenzene sulfonic acid, α-naphthylamine and *n*-butyl alcohol were added and the final supernatant was used for measuring absorbance at 530 nm.

The concentrations of MDA were determined following the method proposed by Hodges et al. (1999) with the corrected equation proposed by Landi (2017) and concentrations of hydroperoxide conjugated diene (HPCD) were obtained from frozen leaves in ethanol (96%) by spectrophotometric UV–Vis method. The absorbance was measured at 234 nm (Levin and Pignata, 1995).

Further analytical details about ROS and indicators of oxidative stress can be found in Esposito et al. (2018).

### 2.5. Statistics

The significant differences between the treatments relative to fluorescence emission of chloroplast metabolites, physiological and biochemical responses and relative growth rates between the initial and final measurements (T3 − T0) were determined by one-way ANOVA. When necessary, the data were transformed to reach normal distribution and equal variances. The Holm-Sidak method was employed to identify significant differences between the three treatments (AA, AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0). The significant differences in monthly relative growth rates (RGR) were tested by two-way ANOVA with repeated measures (factor 1: $O_3$ treatment; factor 2: measurement time). After testing the interaction of both factors, the Holm-Sidak method was employed to identify significant differences between the three treatments and different measurement times. Results were considered significant at $p$ b 0.05.

## 3. Results

### 3.1. Environmental conditions during the experimental period

During the experimental period, the average daily (24 h) air temperature varied between 19 and 32 °C and daily maximum hourly values varied between 21 and 43 °C (Fig. 1). Average daily GSR was 57–400 W $m^{-2}$ and daily maximum hourly values were 927–1186 W $m^{-2}$. Total daily precipitation varied between 0 and 62 mm, and average daily relative humidity was 23–85%. The mean daily $O_3$ concentrations (24 h) varied between 17 and 71 ppb at AA, 23 and 91 ppb at AA + $O_3$ × 1.5 ppb and 28 and 111 ppb at AA + $O_3$ × 2.0 (Fig. 1). After 97 days of exposure, AOT40 reached 22, 41 and 58 ppm h at AA, AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0 treatments, respectively.

### 3.2. Anatomical responses

The asymptomatic leaf blade of AA plants showed uniseriated epidermis and thin cuticle. The leaf blade had a uniseriate hypostomatic epidermis and a thin cuticle. The mesophyll was dorsiventral with one layer of palisade parenchyma and 4–5 layers of spongy parenchyma cells. The parenchyma cells showed thin cell walls, peripheral flattened chloroplasts with starch grains, hyaline vacuole and pronounced intercellular spaces (Fig. 2A–D). A strong reaction to total proteins stain was observed in the chloroplasts (Fig. 2E).

Symptomatic leaves from intermediate and elevated levels of $O_3$ (AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0, respectively) showed changes in cell wall of the palisade parenchyma cells, that exhibited a sinuous shape (Fig. 2G). An apparent reduction in the size and density of chloroplasts and changes in their shapes was observed in the palisade cells along the leaf blade (Fig. 2F–H). In addition, the starch grains inside the

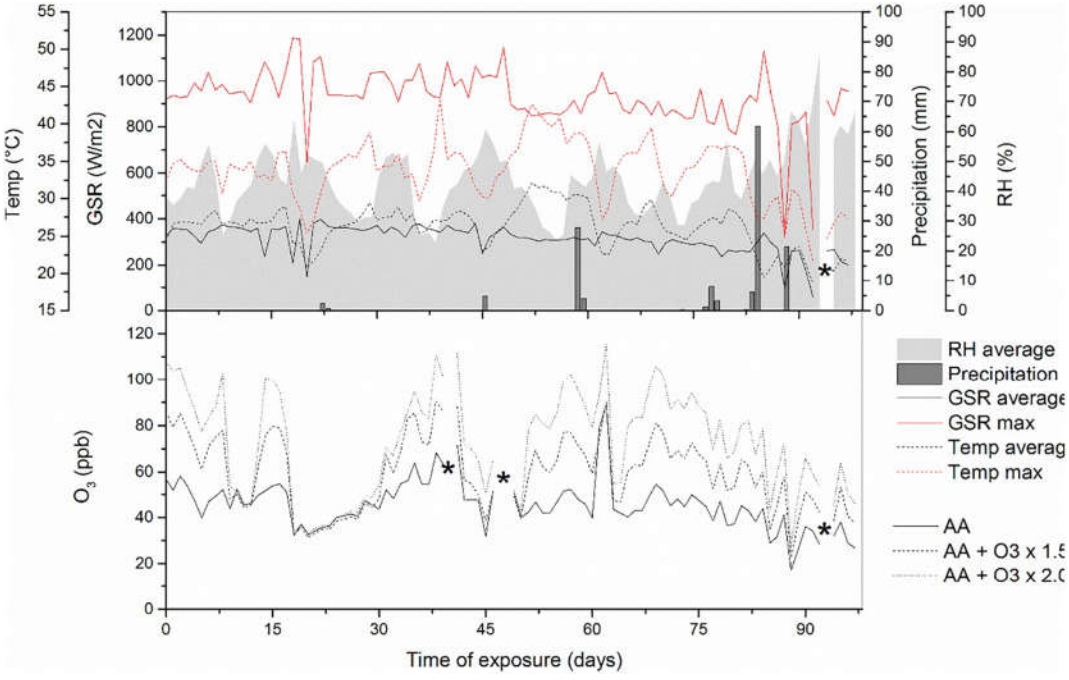



Fig. 1. Environmental conditions over the experimental period (from June 10th to September 15th, 2017 = 97 days of exposure). (*) indicates the absence of data. Daily average of temperature (Temp average), relative humidity (RH average), global solar radiation (GSR average), ozone concentrations at ambient air (AA), intermediate ozone level (AA + $O_3$ × 1.5) and elevated ozone level (AA + $O_3$ × 2.0) and total daily precipitation (P). Daily maximum of temperature (Temp max) and global solar radiation (GSR max).

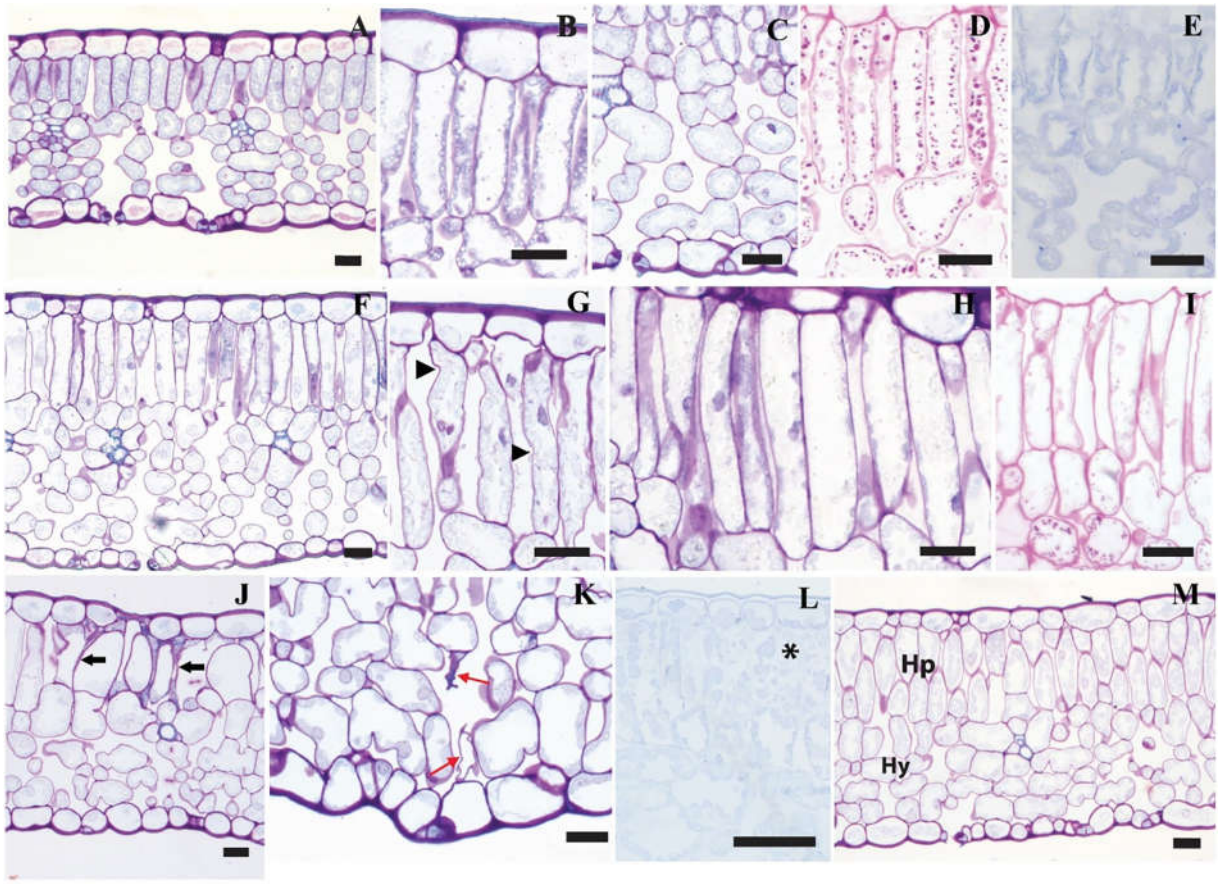



Fig. 2. Structural aspects of *P. edulis* leaves from ambient air (A–E) and from ozone treatments (AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0) (F–M). Parenchyma cells with thin cell walls, hyaline vacuole and pronounced intercellular spaces (A). Flattened chloroplasts diffusely distributed in the palisade parenchyma (B) and spongy parenchyma cells (C). Starch grains inside the chloroplasts (D). A strong reaction to total proteins stain inside the chloroplasts (E). An apparent reduction in the amount, size and density of chloroplasts along the leaf blade (F–H). Changes in cell wall and chloroplasts shape (arrowheads, G). Chloroplasts do not accumulate starch grains (I). Collapse of palisade (arrows, J) and spongy parenchyma cells close to stomata (red arrows, K). Weak reaction to protein stain. Proteins accumulated in the protoplast (black asterisk, L). Hyperplasia of palisade parenchyma (Hp) and hypertrophy (Hy) of spongy parenchyma cells in AA + $O_3$ × 2.0 (M). Toluidine blue (A–C, F–H, J–L, M); PAS test, total polysaccharides (D and I); Coomassie blue, total proteins (D and L). Bars = 25 μm.

chloroplasts in the parenchyma palisade were smaller or absent (Fig. 2I). Small groups of collapsed cells were observed in the palisade (Fig. 2J) and spongy parenchyma (Fig. 2K). A weaker reaction to protein stain and a positive reaction in the protoplast were observed in $O_3$–exposed leaves compared to AA (Fig. 2E vs L).

The symptomatic leaves from AA + $O_3$ × 2.0 showed reductions of intercellular spaces resulting from hyperplasia of palisade parenchyma, identified as an increase in the number of cell layers, and hypertrophy of spongy parenchyma, characterized by an increase in its cell size (Fig. 2O).

Asymptomatic leaves exhibited low emission intensity of constitutive flavonoids located in the outer envelope membrane (OEM) and of carotenoids, with the emission intensity of carotenoids higher than that of OEM-flavonoids (Fig. 3A). There was also a low emission intensity of pheophytins (Fig. 3A). In addition, a high emission intensity of chlorophylls (N650 nm) was observed regardless of the region of interest (ROI) selected for analysis (Fig. 3B). In contrast, symptomatic samples from ozone treatments (AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0) had an increase of the emission intensity of flavonoids, carotenoids, pheophytins and lipofuscins-like (Fig. 3C). The chlorotic cells did not exhibit chlorophyll emission, while adjacent chlorotic cells (ROI 2) exhibited a similar emission intensity pattern as the one observed in the AA samples (Fig. 3D). The emission intensity of lipofuscin-like and OEM flavonoids in the chloroplasts were significantly higher in the samples from treatments with intermediate and elevated levels of $O_3$ when compared to those from the AA treatment (Fig. 3E). Although the emission intensity of carotenoids in leaf samples from the treatments with ozone was higher than that obtained in the AA treatment, only the results from the AA + $O_3$ × 1.5 differed significantly from the AA treatment (Fig. 3E). There were no significant differences between treatments in terms of chlorophyll and pheophytin emission intensities (Fig. 3E).

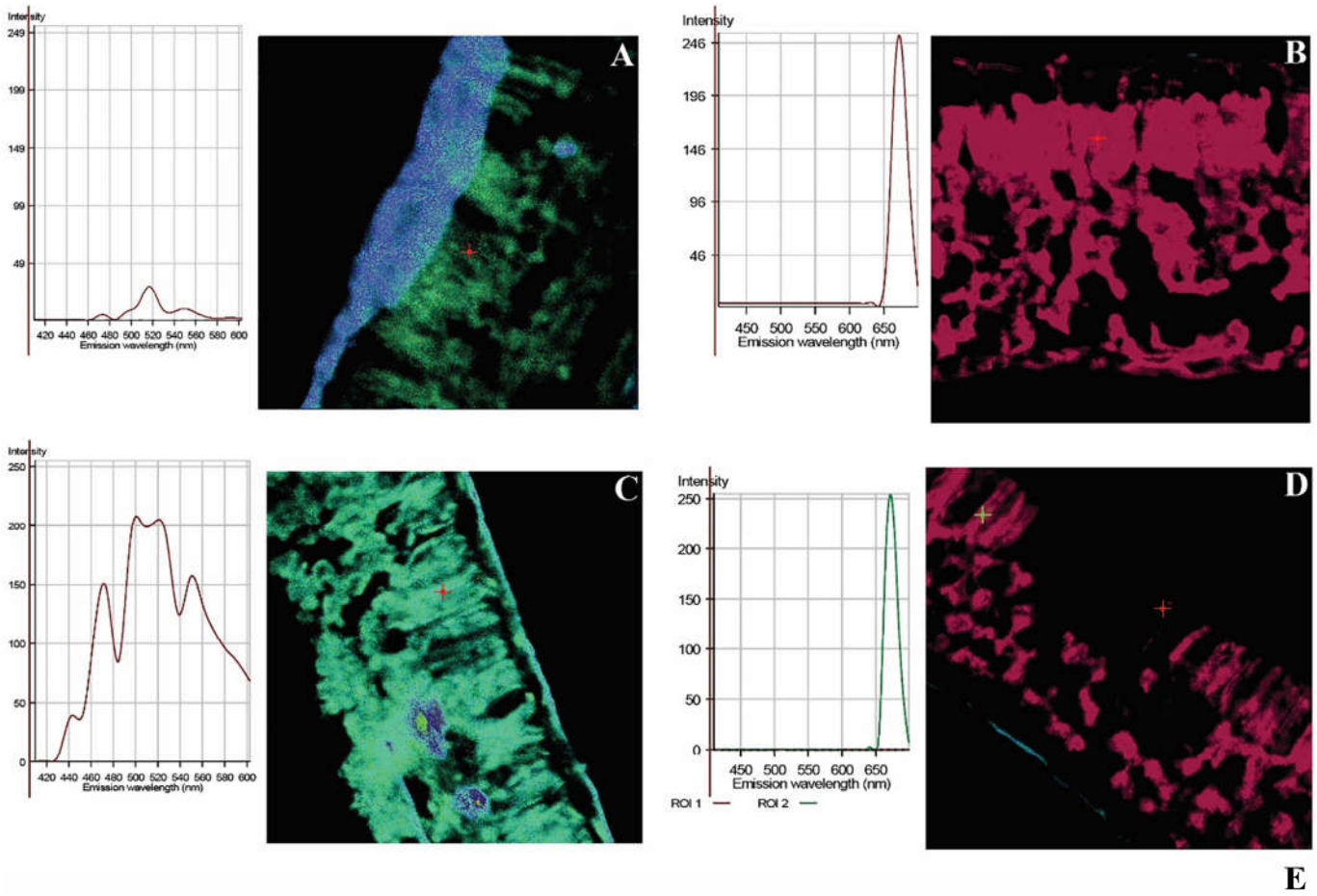
### 3.3. Physiological and biochemical responses

#### 3.3.1. Leaf gas exchange

One-way ANOVA revealed that the light-saturated net photosynthetic rate ($A_{sat}$) of *P. edulis* leaves did not differ significantly among $O_3$ treatments (Table 1). Stomatal conductance ($g_{sw}$) and the Ci/Ca ratio were not statistically different among $O_3$ treatments. PODo varied between 13.52 mmol $m^{-2}$ at AA, 17.24 mmol $m^{-2}$ at AA + $O_3$ × 1.5 and 20.62 mmol $m^{-2}$ at AA + $O_3$ × 2.0 treatments.

#### 3.3.2. Relative growth rates

The interacting effects of both factors ($O_3$ treatment and time of measurement) on the relative growth rates (RGR) were not significant for any growth parameter ($p$ N 0.05). The elevated $O_3$ level (AA + $O_3$ × 2.0) reduced the RGR in leaf number during all time intervals in comparison to the other treatments. No significant effect of $O_3$ was proved on the RGR in diameter. However, in relation to RGR in height, significant differences were found among times of measurement. In general, lower RGRs were observed during the last month of experiment (T3 and T2) for all parameters (Fig. 4).




| treatment | lipofuscins (intensity- nm) | flavonoids (intensity- nm) | carotenoids (intensity -nm) | pheophytins (intensity- nm) | clorophyll (intensity- nm) |
|---|---|---|---|---|---|
| AA | 0.77 (±1.13) b | 18.01 (± 16.38) b | 60.38 (±22.61) b | 20.63 (± 16.21) a | 107.83 (± 14.32) a |
| AA + $O_3$ x 1.5 | 5.38 (± 0.98) a | 75.52 (± 9.88) a | 126.35 (± 16.67) a | 57.44 (± 13.28) a | 54.76 (± 38.09) a |
| AA + $O_3$ x 2.0 | 3.42 (± 1.05) a | 55.72 (± 16.77) a | 108.5 (± 22.87) ab | 46.58 (± 21.85) a | 70.96 (± 20.59) a |

Fig. 3. Emission spectra of chloroplasts (A–D) and quantification of the emission intensity of chloroplast metabolites (E) in leaves of *P. edulis* from ambient air (AA) (A, B and E) and ozone treatments (AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0) (C, D and E). Emission spectra of chloroplasts from AA (regions of interest; ROI 1 in the palisade parenchyma): the peak between 450 and 500 nm refers to flavonoids, 500 and 550 nm to carotenoids and 550 and 600 pheophytins (A). Emission referring only to chlorophyll (above 650 nm; ROI 1 in the palisade parenchyma) from AA (B). Emission spectra of chloroplasts from ozone treatments (C). Note that the peaks related to carotenoids (500–550 nm), flavonoids (450–500 nm) and pheophytins (550–600 nm) increase and appears lipofuscin-like peaks (peak between 400 and 450 nm refers to lipofuscin). Emission referring only to chlorophyll in the palisade parenchyma from ozone treatments (D). Note the absence of chlorophyll emission from some groups of palisade parenchyma cells (ROI 1), while adjacent cells (ROI 2) exhibited. Median values (± standard deviation) of the intensity of lipofuscins, flavonoids, carotenoids, pheophytins and chlorophyll fluorescence emission peaks from chloroplasts (E). Different letters indicate significant differences in each treatment ($p$ b 0.05, Holm-Sidak method test, N = 3).

Table 1
Photosynthetic traits ($A_{sat}$, light-saturated net photosynthetic rate; $g_{sw}$, stomatal conductance for water vapour; Ci/Ca, ratio of intercellular $CO_2$ concentration (Ci) to ambient $CO_2$ concentration) of leaves of *P. edulis* seedlings exposed to three treatments of ozone (AA = ambient air, AA + $O_3$ × 1.5 = intermediate and AA + $O_3$ × 2.0 = elevated ozone levels) for 97 days. Data are shown as mean ± S.E. (n = 3). Different letters indicate significant differences between the treatments by Holm-Sidak test ($p$ b 0.05), n = 3.

| Treatment | $A_{sat}$ (μmol $m^{-2}$ $s^{-1}$) | $g_{sw}$ (mol $m^{-2}$ $s^{-1}$) | Ci/Ca ratio (fraction) |
|---|---|---|---|
| AA | 8.8 ± 0.1 a | 0.10 ± 0.01 a | 0.58 ± 0.03 a |
| AA + $O_3$ × 1.5 | 7.7 ± 0.5 a | 0.07 ± 0.01 a | 0.49 ± 0.03 a |
| AA + $O_3$ × 2.0 | 6.9 ± 0.6 a | 0.07 ± 0.01 a | 0.50 ± 0.04 a |

The biomass of leaf, stem and root as well as the shoot to root ratios did not vary significantly among the $O_3$ treatments (data not shown).

### 3.3.3. *Pigments*

The chlorophyll *a* content, chlorophyll *a*/*b* ratio and carotenoid content were significantly higher in the treatment with elevated levels of ozone than in AA and AA + $O_3$ × 1.5 (Table 2).

### 3.3.4. *Primary and secondary metabolites*

The leaf content of total sugars did not differ among treatments and the leaf content of starch was significantly higher in the AA + $O_3$ × 1.5 treatment than in the others (Table 2).

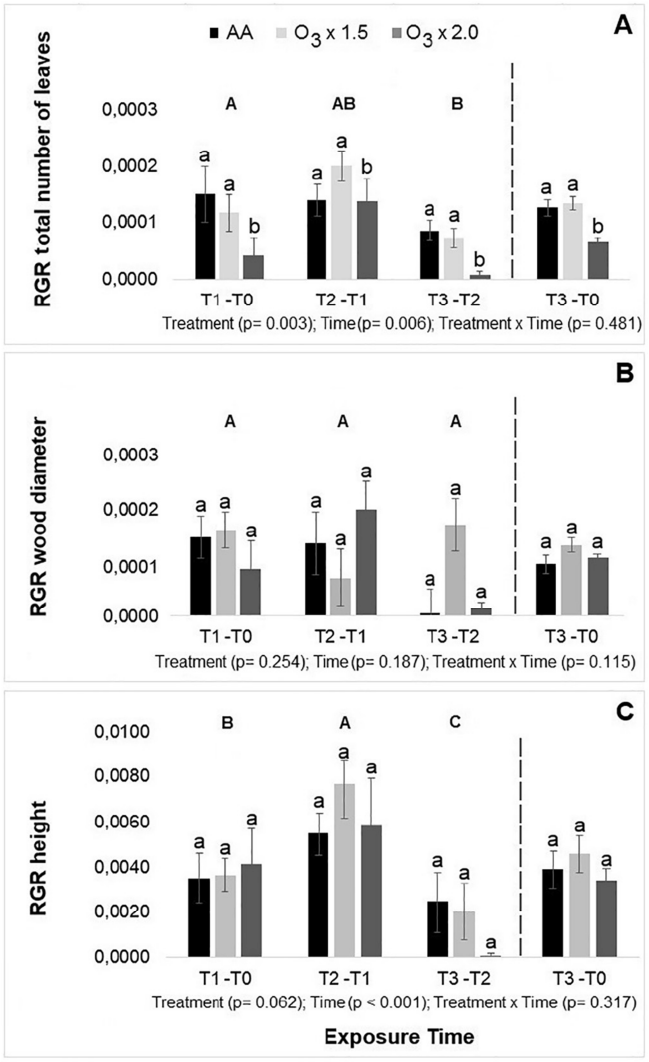



Fig. 4. Relative growth rates (RGR) per day of fumigation between monthly intervals of measurement (T1 – T0, T2 – T1, T3 – T2) and between the initial and final measurements (T3 – T0), of: total number of leaves (A), diameter (B) and height (C) of *P. edulis* plants exposed to three treatments of ozone (AA = ambient air, AA + $O_3$ × 1.5 = intermediate and AA + $O_3$ × 2.0 = elevated ozone levels) for 97 days. Different lower case letters indicate significant differences between the treatments during the same monthly interval of measurement. Different upper case letters indicate differences in the RGR between monthly intervals of measurement in the same treatment. p b 0.05, Holm-Sidak method, N = 3.

Leaf accumulation of total flavonoids was higher in plants exposed to AA + $O_3$ × 2.0 and to AA + $O_3$ × 1.5 than in plants from AA.

### 3.3.5. *Antioxidants*

The content of non-enzymatic antioxidants – ascorbic acid and glutathione in their reduced, oxidized and total forms – was significantly enhanced in the treatments with intermediate and elevated levels of ozone when compared to AA treatment (Table 2). There was no difference between the ozone treatments (×1.5 and ×2.0). The redox potential of both ascorbic acid (medians ≥ 0.71) and glutathione (medians ≥ 0.48) was high but did not differ between the ozone treatments. The activity of enzymatic antioxidants – CAT, GR and APX – was significantly higher in the plants grown in the AA treatment than in the plants included in the treatments with ozone addition. The activity of SOD did not differ among the treatments (Table 2).

### 3.3.6. *Reactive oxygen species and lipid peroxidation derivatives*

The leaf accumulation of •OH was similar in plants from all treatments, but the $O_2^{\bullet -}$ ion and the $H_2O_2$ content were higher in the treatments with intermediate and elevated levels of ozone when compared to AA.

The indicators of lipid peroxidation – MDA and HPCD – and flavonoids also increased in the plants included in the treatments with intermediate and elevated levels of ozone when compared to AA treatment (Table 2).

## 4. Discussion

The occurrence of symptomatic leaves indicated that *P. edulis* plants exposed to high levels of ozone showed injury of leaf cells and tissues. The visible $O_3$ symptoms were restricted to foliar yellowing, which is generally a consequence of chlorophyll degradation (Günthardt-Goerg and Vollenweider, 2007). The appearance of visible injury is a common symptom observed in vine species exposed to $O_3$. It was registered for example by Saitanis (2003) in *Vitis vinifera* in Greece, by Manning and Godzik (2004) in *Humulus lupulus* in Central and Eastern Europe and by Ferreira et al. (2012) in *Ipomoea nil* 'Scarlet O'Hara' in the state of São Paulo, Brazil. The confocal analyses indicated a degradation of chlorophylls inside the chloroplasts of chlorotic leaves under elevated ozone levels, compared to chlorophyll emissions in asymptomatic leaves of plants exposed to ambient ozone level. However, such difference was not statistically significant. Peaks related to products of oxidative damage were observed by confocal analyses (lipofuscin-like and pheophytins). Pheophytins are the first product resulting from the degradation of chlorophyll under air pollution (Gowin and Goral, 1977), characterized by the loss of the central Mg of the chlorophyll molecule (Eijckelhoff and Dekker, 1997). Although pheophytin emission increased in symptomatic leaf samples under the ozone treatments, the chlorophyll emission under confocal analysis did not change significantly. The confocal analysis does not distinguish chlorophyll *a* and *b*, as well as pheophytin *a* and *b*. The chlorophyll analysis from asymptomatic leaves revealed an increase in the concentration ratio between chlorophylls *a* and *b* due to enhanced levels of chlorophyll *a* in plants exposed to the highest level of ozone, which explains the non-significant variation in chlorophyll emission in the confocal analyses. These results indicated the conversion of chlorophyll *b* to pheophytins *b* (Xu et al., 2001). In addition, the chl *a*/*b* ratio is very low as compared to commonly observed values in higher plants.

The foliar yellowing and chlorophyll alterations can be indicative of premature foliar senescence in response to ozone (Pell et al., 1997). Accelerated senescence in response to $O_3$ exposure was observed in the vine *Vitis vinifera* in controlled-exposure experiments (Soja et al., 1997). Accelerated foliar senescence may be one of the explanations for the significantly lower leaf number of *P. edulis* plants under the highest level of $O_3$. This effect might be considered an avoidance mechanism that restrains the progression of $O_3$ damage in the plants. This mechanism, together with the anatomical, biochemical and physiological responses discussed below, may contribute to improve $O_3$ tolerance in *P. edulis*, as suggested by no significant effect on biomass.

*P. edulis* mesophyll cells showed typical structural markers of accelerated cell senescence (ACS) in response to $O_3$, such as chloroplast degeneration, reduced chloroplast size or irregular shape (Günthardt-Goerg and Vollenweider, 2007) and decreased protein cell content (Günthardt-Goerg et al., 1997; Günthardt-Goerg and Vollenweider, 2007; Vollenweider et al., 2013; Moura et al., 2018). The proteins accumulated in the chloroplasts and protoplast suggest that they may be enzymes linked to senescence processes, since hydrolytic enzymes are involved in the degradation of cellular components in senescent leaves (Diaz-Mendoza et al., 2016).

Reduction in number and size, and degradation of starch grains in the chloroplasts induced by ozone were also reported in other controlled experiments and were associated with accelerated senescence

Table 2
Mean, median and range between minimum and maximum values of biochemical markers in leaves of *P. edulis* exposed to three treatments of ozone (AA = ambient air, AA + $O_3$ × 1.5 = intermediate and AA + $O_3$ × 2.0 = elevated ozone levels) for 97 days: reduced (AsA), oxidized (DHA), total (totAA) ascorbic acid (mg/g$^{-1}$ dw), redox potential of ascorbic acid (AsA/AsA + DHA), reduced (GSH), oxidized (GSSG), total (totG) glutathione (μmol/g$^{-1}$dw), redox potential of glutathione (GSH/GSH + GSSG), catalase (CAT, unit/g$^{-1}$dw), glutathione reductase (GR, unit/g$^{-1}$ dw), ascorbate peroxidase (APX, unit/g$^{-1}$ dw) superoxide dismutase (SOD, unit/g$^{-1}$ dw), total sugars (mg/g$^{-1}$ dw), starch (mg/g$^{-1}$ dw), total flavonoids (%), carotenoids (CAR, mg/g$^{-1}$ dw), chlorophylls *a*, *b* and *total* (Chl *a*, Chl *b*, Chl *total*, mg/g$^{-1}$ dw), chlorophyll ratio (Chl *a*/*b*), hydroxyl radical (·OH, % 2′-deoxyribose oxidative degradation dw), superoxide ($O_2$·$^-$, nmol/g dw), hydrogen peroxide ($H_2O_2$, μmol/g dw), malondialdehyde (MDA, mM$^{-1}$ dw) and hydroperoxide conjugated diene (HPCD μmol/g$^{-1}$ dw). Different letters indicate significant differences between the treatments for each parameter median by Holm-Sidak test ($p$ b 0.05), n = 3.

| Biochemical markers | AA | | AA + $O_3$ × 1.5 | | AA + $O_3$ × 2.0 | |
|---|---|---|---|---|---|---|
| | Mean | Range | Mean | Range | Mean | Range |
| *Pigments* | | | | | | |
| Chl *a* | 0.50 b | 0.38–0.63 | 0.56 b | 0.38–0.71 | 0.63 a | 0.48–0.75 |
| Chl *b* | 0.81 a | 0.55–0.99 | 0.83 a | 0.53–1.06 | 0.67 a | 0.39–0.92 |
| Chl *total* | 1.31 a | 0.98–1.58 | 1.39 a | 0.99–1.68 | 1.30 a | 0.94–1.68 |
| Chl *a*/*b* | 0.62 b | 0.56–0.78 | 0.70 b | 0.36–0.97 | 0.97 a | 0.73–1.38 |
| CAR | 0.03 b | 0.01–0.03 | 0.03 b | 0.01–0.06 | 0.06 a | 0.04–0.06 |
| *Primary and secondary metabolites* | | | | | | |
| Total sugars | 86.76 a | 70.06–120.98 | 101.70 a | 83.41–139.58 | 101.16 a | 82.24–118.65 |
| Starch | 52.19 b | 29.46–77.06 | 73.97 a | 52.80–86.60 | 48.02 b | 34.78–90.66 |
| Total flavonoids | 0.20 c | 0.18–0.21 | 0.28 b | 0.27–0.29 | 0.32 a | 0.31–0.33 |
| *Antioxidants* | | | | | | |
| AsA | 1.51 b | 1.24–2.37 | 2.14 a | 1.50–2.83 | 3.03 a | 2.61–3.34 |
| DHA | 0.62 b | 0.22–1.80 | 0.86 a | 0.25–1.63 | 0.50 a | 0.15–1.02 |
| totAA | 2.13 b | 1.55–3.33 | 3.00 a | 2.80–3.47 | 3.53 a | 3.17–3.84 |
| AsA/AsA + DHA | 0.73 a | 0.45–0.89 | 0.71 a | 0.47–0.91 | 0.85 a | 0.73–0.95 |
| GSH | 23.56 b | 11.93–38.96 | 32.76 a | 20.33–47.06 | 54.60 a | 31.14–114.26 |
| GSSG | 22.19 b | 8.99–35.70 | 31.07 a | 17.34–44.60 | 52.53 a | 30.81–94.61 |
| totG | 45.75 b | 20.92–74.67 | 63.83 a | 54.73–79.47 | 107.13 a | 61.95–208.88 |
| GSH/GSH + GSSG | 0.57 a | 0.39–0.75 | 0.51 a | 0.35–0.70 | 0.50 a | 0.37–0.56 |
| CAT | 206.33 a | 129.98–317.85 | 179.89 b | 119.65–244.10 | 170.72 b | 135.00–194.88 |
| GR | 0.83 a | 0.10–1.70 | 0.28 b | 0.08–0.60 | 0.21 b | 0.05–0.53 |
| APX | 24.17 a | 13.42–39.54 | 10.95 b | 4.49–24.84 | 12.61 b | 3.90–29.14 |
| SOD | 0.24 a | 0.10–0.49 | 0.27 a | 0.16–0.38 | 0.13 a | 0.01–0.33 |
| *Reactive oxygen species* | | | | | | |
| ·OH | 47.27 a | 44.19–50.20 | 49.32 a | 42.26–53.97 | 47.71 a | 40.02–54.48 |
| $O_2$·$^-$ | 26.14 b | 20.25–36.75 | 59.09 a | 44.61–69.00 | 68.37 a | 33.91–87.00 |
| $H_2O_2$ | 65.29 b | 58.11–69.77 | 76.01 a | 58.84–96.94 | 76.42 a | 71.17–86.63 |
| *Lipid peroxidation* | | | | | | |
| MDA | 18.47 b | 13.56–24.18 | 22.27 b | 12.86–35.03 | 28.46 a | 22.01–37.82 |
| HPDC | 0.62 b | 0.55–0.78 | 0.70 b | 0.36–0.97 | 0.97 a | 0.73–1.38 |

(Bäck et al., 1999; Moura et al., 2018), and with the decline in the carboxylation efficiency (Oksanen et al., 2001). The reduction of starch grains in ozone-fumigated leaves suggest that less carbon was available for tissue repairing, production of antioxidants and growth (Bäck et al., 1999). However, we observed few alterations in the starch contents of the leaf tissues under high levels of ozone and we did not observe changes in the content of total sugars and in net photosynthesis and biomass production, indicating that changes in the structure of starch grains inside the chloroplasts did not affect the associated physiological processes.

The partial or total collapse of groups of cells frequently observed in the palisade parenchyma was also indicative of the programmed cell death (PCD) process following the ACS induced by $O_3$ in *P. edulis*. Increased ROS concentrations (as observed for hydrogen peroxide and superoxide in the present study) can cause rapid localized cell death, characterized by incomplete cellular degradation, altered cell membrane integrity and cell wall changes leading to cell collapse, processes that do not require energy (Günthardt-Goerg and Vollenweider, 2007). These cellular alterations indicate hypersensitive response-like (HR-like, similar to those induced by biotic stress) commonly found in $O_3$ fumigated species, including native vine species (Moura et al., 2011; Alves et al., 2016). We suggest that the deformation of the chloroplasts and the sinuous cell walls of palisade parenchyma were the first stages in the cell death process that culminated in the total collapse of groups of cells. In addition, cell collapse in the spongy parenchyma was also observed in the present study, although less frequently, mainly around the stomata. The same evidences were observed in the vine species *Ipomoea nil* under controlled conditions (Moura et al., 2011).

Hypertrophy and hyperplasia of the mesophyll cells were observed in studies that simulated the effect of acid rain on leaves (Silva et al., 2005; Sant'Anna-Santos et al., 2006). Although this is unusual, hypertrophy may also occur in the mesophyll in response to $O_3$ (Fink, 1999). We suggest that the hypertrophy and hyperplasia observed in mesophyll cells in *P. edulis* indicated acclimation to ozone. Both phenomena increase the compactness of the mesophyll layer, thus increasing the resistance to ozone diffusion. In addition, the increase of mesophyll compactness resulted in a higher leaf mass per area in leaves belonging to AA + $O_3$ × 2.0 treated plants, which can explain unchanged levels of biomass production among treatments in concomitance with reduction of RGR of leaves under high ozone.

Lipofuscin-like compounds were detected by confocal analyses in higher proportion inside the chloroplasts of leaf tissues from the high ozone treatment than in those from the AA treatment. Lipofuscins are liposoluble fluorescent products originating from the interaction of malonyl-dialdehyde with protein amino groups (Roshchina and Roshchina, 2003; Roshchina et al., 2015). Peak emission of this lipid peroxidation indicator was evidenced in pollen of *Passiflora caerulea* after 0.15 ppm–5 ppm h $O_3$ doses (Roshchina and Mel'nikova, 2001). In addition, the increased contents of MDA and HPDC in leaves from the $O_3$ × 2.0 treatment suggest that the lipid peroxidation resulted in ACS. In fact, these compounds are produced at the beginning and end of the lipid peroxidation chain, respectively. The ACS can be accelerated by an early onset of visible injury and also occurs in younger tissues, in response to increased ROS concentrations (Günthardt-Goerg and Vollenweider, 2007; Alves et al., 2016).

The increased hydrogen peroxide and superoxide leaf concentrations might also have stimulated antioxidant responses in *P. edulis* exposed to ozone, such as enhancement in the levels of flavonoids, ascorbic acid, glutathione and carotenoids. The constitutive flavonoids (of C-glycoside type) are one of the main bioactive compounds found in leaves of *P. edulis* (Ayres et al., 2015). We can assume that a great part of the increase in the leaf content of flavonoids came from the chloroplasts, based on the direct observation of in situ emission of these compounds by confocal laser scanning microscopy. The flavonoids located in the outer envelope membranes of the chloroplast (OEM) have antioxidant functions, limit the diffusion of ROS out of the chloroplast and preserve the outer membrane against oxidative damage (Roshchina and Roshchina, 2003; Agati et al., 2007, 2012). Besides, the biosynthesis of some flavonoids occurs inside of the chloroplasts (Agati et al., 2007).

Plants are equipped with an efficient antioxidant apparatus composed by antioxidant and non-enzymatic components which balance the cellular redox homeostasis and protect them against excessive ROS-triggered oxidative stress. The ascorbate-glutathione cycle ensures the regeneration of ascorbate by a sequence of redox reactions involving glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) (Foyer and Noctor, 2011). The antioxidant power is not only limited to ascorbate and glutathione but also the antioxidant compounds such as superoxide dismutase, peroxidases and catalases (Caregnato et al., 2008). Under favorable conditions, a fully-functional antioxidant machinery maintains the redox balance with plant cells. However, enhancement of ROS generation promoted by different environmental cues, such as ozone, can lead to the alteration of redox balance, this promoting oxidative stress events (Bray et al., 2000).

In addition to ROS scavenging promoted by ascorbate-glutathione components, other secondary metabolites with a strong free-radical-scavenging ability can sensibly participate in ROS controlling in leaves, these including flavonoids (Agati et al., 2013).

A decrease in the activity of APX in the chloroplasts may be followed by an increase in the levels of flavonoids to control ROS propagation. This response was observed in the plants of *P. edulis* exposed to high ozone concentrations. Flavonoids like rutin and quercetin are also scavengers of superoxide anions (Yuting et al., 1990) and for this reason they may act in place of SOD thereby explaining the decrease of the activity of this enzyme in leaf samples of *P. edulis*. SOD catalyzes the dismutation of the superoxide radical in $H_2O_2$ and $H_2O$ in the presence of proton $H^+$ (Scandalios, 1993). In foliar samples of *P. edulis* the increment of $H_2O_2$ suggests the inhability of CAT and APX to control the level of $H_2O_2$ generated by the superoxide anion scavenging ability orchestrated by flavonoids and SOD activity. Flavonoids are capable of inhibiting glutathione reductase (Elliot et al., 1992) which also could explain the decrease of activity of this enzyme in leaf samples of *P. edulis*. Although some studies (e.g. Furlan et al., 2010; Santos and Furlan, 2013) reported an increase in the concentrations of flavonoids in plants exposed to ozone, none of them related this enhancement with the induction of OEM flavonoids. Foliar levels of other non-enzymatic antioxidants (ascorbic acid and glutathione) were enhanced in plants exposed to the AA + $O_3$ × 1.5 and AA + $O_3$ × 2.0 treatments, respectively, when compared to plants grown in AA. The high leaf contents of ascorbic acid and glutathione, as well as their high and stable redox potential, as indicated by the ratio between their reduced and total forms (AsA/AsA + DHA; GSH/GSH + GSSG), were key responses for maintaining high redox equilibrium in *P. edulis* under ozone, although enhanced lipid peroxidation or MDA by-products were observed in plants exposed to the highest ozone concentrations. In our experiment the increment of AsA and the higher AsA/AsA + DHA suggest that *P. edulis* plants were able to efficiently regenerate the oxidized DHA to reduced AsA which is the biological active form of ascorbate capable of ROS scavenging (Burkey et al., 2006), this support the $O_3$ tolerance of *P. edulis*. Similar results were obtained for other species, under either experimental or natural conditions (e.g. Burkey et al., 2006; Aguiar-Silva et al., 2016; Esposito et al., 2016; Brandão et al., 2017). The high levels of carotenoids, observed inside the chloroplasts in plants fumigated with high ozone, can be also considered biochemical responses associated to the high tolerance of *P. edulis* against environmental oxidative stressors. Carotenoids are natural pigments mostly responsible for the yellow, orange and red color of the fruits (Da Silva et al., 2014), being z-carotene identified as the predominant compound in passion fruit (Pertuzatti et al., 2015). Carotenoids are essential for the correct assembly and functioning of photosystems and protect from photo-oxidative damage preventing and quenching ROS generated from triplet excited chlorophylls via xanthophyll cycle (Esteban et al., 2015). While non-enzymatic antioxidants generally showed higher concentration in the ozone-addition treatments, the enzymatic activity of CAT, APX and GR decreased at the elevated ozone treatment ($O_3$ × 2.0). The lowest activity level of these enzymes in plants from this treatment coincided with the highest contents of ascorbic acid and glutathione, showing that the ascorbate–glutathione cycle of *P. edulis* was stimulated in response to $O_3$. Dafré-Martinelli et al. (2011) analyzed the redox state of the vine *Ipomoea nil* 'Scarlet O'Hara' growing under $O_3$ in an urban area in Brazil, and concluded that ascorbic acid and glutathione were crucial for increasing plant tolerance to ozone.

## 5. Conclusions

Exposure to high levels of $O_3$ did not significantly affect the content of total sugars, net photosynthesis, growth parameters (diameter and height) and biomass production. *P. edulis* showed several reactive mechanisms against the effects of $O_3$, such an effective enhancement antioxidant system, principally consisting of non-enzymatic antioxidants (ascorbic acid, carotenoids, glutathione and flavonoids located in the outer envelope membranes of the chloroplast), hyperplasia and hypertrophy of the mesophyll cells (thus reducing the intercellular space and increasing the resistance to ozone diffusion), and accelerated cell senescence which may have accelerated leaf abscission and thus reduced the number of leaves per plant. So, these leaf traits indicate *P. edulis* as tolerant to the oxidative stress caused by $O_3$.

## AcknowledgThents

The authors thank the financial support provided by Fundação de Desenvolvimento da Pesquisa do Agronegócio (FUNDEPAG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fondazione Cassa di Risparmio di Firenze (2013/7956) and the LIFE15 ENV/IT/000183 project MOTTLES. We also thank Alessandro Materassi for design and maintenance of the ozone FACE, Moreno Lazzara for assistance during the field work, Adriana Matsukuma and Wilton Lima for assistance with confocal microscopy at Central Analítica (Instituto de Química, USP).

## Appendix A. SuppleThentary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.425.

## References

Agati, G., Matteini, P., Goti, A., Tattini, M., 2007. Chloroplast-located flavonoids can scavenge singlet oxygen. New Phytol. 174, 77–89.

Agati, G., Azzarello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: location and functional significance. Plant Sci. 196, 67–76.

Agati, G., Brunetti, C., Ferdinando, M., Ferrini, F., Pollastri, S., Tattini, M., 2013. Functional roles of flavonoids in photoprotection: new evidence, lessons from the past. Plant Physiol. Biochem. 72, 35–45.

Aguiar-Silva, C., Brandão, S.E., Domingos, M., Bulbovas, P., 2016. Antioxidant responses of Atlantic Forest native tree species as indicators of increasing tolerance to oxidative stress when they are exposed to air pollutants and seasonal tropical climate. Ecol. Indic. 63, 154–164.

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 24, 1337–1344.
Alves, E.S., Moura, B.B., Pedroso, A.N.V., Tresmondi, F., Machado, S.R., 2016. Cellular markers indicative of ozone stress on bioindicator plants growing in a tropical environment. Ecol. Indic. 67, 417–424.
Amaral, L.I.V., Costa, P.M.F., Aidar, M.P.M., Gaspar, M., Buckeridge, M.S., 2007. Novo método enzimático rápido e sensível de extração e dosagem de amido em materiais vegetais. Hoehnea 34, 425–431.
Amorim, T.A., Nunes-Fretiras, A.F., Rosado, B.H.P., 2018. Revisiting the hypothesis for increasing liana abundance in seasonal forest: a theoretical review. Plant Soil 430, 1–6.
Araújo, M.H., Silva, I.C.V., Oliveira, P.F., Barreto, A.R.R., Konnod, T.U.P., Esteves, F.A., Bartha, T., Aguire, F.A., Lopes, N.P., Dermenjianf, R.K., Guimarães, D.O., Leala, I.C.R., Lasunskaiab, E.B., Muzitanoa, M.F., 2017. Biological activities and phytochemical profile of *Passiflora mucronata* from the Brazilian restinga. Rev. Bras. Farmacogn. 27, 702–710.
Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell Environ. 28, 949–964.
Ayres, A.S.F.S.J., Araújo, L.L.S., Soares, T.C., Costa, G.M., Reginatto, F.H., Ramos, F.A., Castellanos, L., Schenkel, E.P., Soares-Rachetti, V.P., Zucolotto, S.M., Gavioli, E.C., 2015. Comaprative central effects of the aqueous leaf extract of two populations of *Passiflora edulis*. Rev. Bras. Farmacogn. 25, 499–505.
Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J., 1998. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation in the leaves and roots of wild-type and a catalase deficient mutant of barley. Physiol. Plant. 104, 280–292.
Bäck, J., Vanderklein, D.W., Topa, M.A., 1999. Effects of elevated ozone on $CO_2$ uptake and leaf structure in sugar maple under two light environments. Plant Cell Environ. 22, 137–147.
Benincasa, M.M.P., 1988. Análise de crescimento de plantas: noções básicas. 2º edição. Funep, Jaboticabal, p. 41.
Bhaduri, A.M., Fulekar, M.H., 2012. Assessment of arbuscular mycorrhizal fungi on the phytoremediation potential of *Ipomoea aquatica* on cadmium uptake. 3 Biotech 2, 193–198.
Brandão, S.E., Bulbovas, P., Lima, M.E., Domingos, M., 2017. Biochemical leaf traits as indicators of tolerance potential in tree species from the Brazilian Atlantic Forest against oxidative environmental stressors. Sci. Total Environ. 575, 406–417.
Bray, E.A., Baylei-Serres, J., Werentilnyk, E., 2000. Responses to abiotic stress. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, USA, New York, pp. 1158–1203.
Burkey, K.O., Neufeld, H.S., Souza, L., Chappelka, A.H., Davison, A.W., 2006. Seasonal profiles of leaf ascorbic acid content and redox state in ozone-sensitive wildflowers. Environ. Pollut. 143, 427–434.
Caregnato, F.F., Koller, C.E., MacFarlane, G.R., Moreira, J.C.F., 2008. The glutathione antioxidant system as a biomarker suite for the assessment of heavy metal exposure and effect in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. Mar. Pollut. Bull. 56, 1119–1127.
Castro, P.C.F., Hoshino, A., Silva, J.C., Mendes, F.R., 2007. Possible anxiolytic effect of two extracts of *Passiflora quadrangularis* L. in experimental models. Phytother. Res. 21, 481–484.
CLRTAP, 2017. Mapping critical levels for vegetation, chapter III of manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. UNECE Convention on Long-range Transboundary Air Pollution On web at. www.icpmapping.org, Accessed date: 27 July 2018.
Da Silva, L.M.R., de Figueiredo, E.A.T., Ricardo, N.M.P.S., Vieira, I.G.P., de Figueiredo, R.W., Brasil, I.M., Gomes, C.L., 2014. Quantification of bioactive compounds in pulps and by-products of tropical fruits from Brazil. Food Chem. 143, 398–404.
Dafré-Martinelli, M., Nakazato, R.K., Dias, A.P.L., Rinaldi, M.C.S., Domingos, M., 2011. The redox state of *Ipomoea nil* 'Scarlet O'Hara' growing under ozone in a subtropical area. Ecotoxicol. Environ. Saf. 74, 1645–1652.
D'Andrea, L., Amenos, M., Rodriguez-Concepcion, M., 2014. Confocal Laser Scanning Microscopy detection of chlorophylls and carotenoids in chloroplasts and chromoplasts of tomato fruit. In: Rodriguez-Concepcion, M. (Ed.), Plant Isoprenoids: Methods and Protocols. Springer, New York, pp. 227–232.
DeWalt, S.J., Chave, J., 2004. Structure and biomass of four lowland Neotropical forests. Biotropica 36, 7–19.
Dhawan, K., Dhawan, S., Sharma, A., 2004. Passiflora: a review update. J. Ethnopharmacol. 94, 1–23.
Diaz-Mendoza, M., Velasco-Arroyo, B., Santamaria, M.E., González-Melendi, P., Martinez, M., Diaz, I., 2016. Plant senescence and proteolysis: two processes with one destiny. Genet. Mol. Biol. 39, 329–338.
Dornelas, M.C., Fonseca, T.C., Rodriguez, A.P.M., 2006. Brazilian passion flowers and novel passionate tropical flowering gems. Floriculture, Ornamental and Plant Biotechnology. Global Science Books, London.
Dubois, M., Gilles, A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–355.
Eijckelhoff, C., Dekker, J.P., 1997. A routine method to determine the chlorophyll a, pheophytin a and β-carotene contents of isolated photosystem II reaction center complexes. Photosynth. Res. 52, 69–73.
Elliot, A.J., Scheiber, S.A., Thomas, C., Pardini, R.S., 1992. Inhibition of glutathione reductase by flavonoids: a structure-activity study. Biochem. Pharmacol. 44 (8), 1603–1608.
Esposito, M.P., Pedroso, A.N.V., Domingos, M., 2016. Assessing redox potential capacity of a native tree from the Atlantic Rain Forest in SE–Brazil during the exchange of the power generation source of an oil refinery. Sci. Total Environ. 550, 861–870.
Esposito, M.P., Nakazato, R.K., Pedroso, A.N.V.P., Lima, M.E.L., Figueiredo, M.A., Diniz, A.P., Kozovitz, A.R., Domingos, M., 2018. Oxidant-antioxidant balance and tolerance against oxidative stress in pioneer and non-pioneer tree species from the remaining Atlantic Forest. Sci. Total Environ. 625, 382–393.
Esteban, R., Barrutia, O., Artetxe, U., Fernández-Marin, B., Hernández, A., García-Plazaola, J.I., 2015. Internal and external factors affecting photosynthetic pigment composition in plants: a meta-analytical approach. New Phytol. 206, 268–280.
Feder, N., O'Brien, 1968. Plant microtechnique: some principles and new methods. Am. J. Bot. 55, 123–142.
Fernandes, F.F., Cardoso-Gustavson, P., Alves, E.S., 2016. Synergism between ozone and light stress: structural responses of polyphenols in a woody Brazilian species. Chemosphere 155, 573–582.
Ferreira, M.L., Esposito, J.B.N., Souza, S.R., Domingos, M., 2012. Critical analysis of the potential of *Ipomoea nil* Scarlet O'Hara for ozone biomonitoring in the sub-tropics. J. Environ. Monit. 14, 1959–1967.
Fink, S., 1999. Pathological and regenerative plant anatomy. Encyclopedia of Plant Anatomy vol. XIV/6. Gebrüder Bornträger, Berlin, Stuttgart, pp. 523–527.
Flora do Brasil 2020, d. Jardim Botânico do Rio de Janeiro.Available at:. http://floradobrasil.jbrj.gov.br/, Accessed date: May 2018 (under construction).
Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2–18.
Furlan, C.M., Santos, D.Y.A.C., Motta, L.B., Domingos, M., Salatino, A., 2010. Guava flavonoids and the effects of industrial air pollutants. Atmos. Pollut. Res. 1, 30–35.
Gahan, P.B., 1984. Plant Histochemistry and Cytochemistry. Academic Press, London.
Gowin, T., Goral, I., 1977. Chlorophyll and pheophytins content in needles of different age of trees growing under conditions of chronic industrial pollution. Acta Soc. Bot. Pol. 46, 151–159.
Günthardt-Goerg, M.S., Vollenweider, P., 2007. Linking stress with macroscopic and microscopic leaf response in trees: new diagnostic perspectives. Environ. Pollut. 147, 467–488.
Günthardt-Goerg, M.S., McQuattie, C.J., Scheidegger, C., Rhiner, C., Matyssek, R., 1997. Ozone-induced cytochemical and ultrastructural changes in leaf mesophyll cell. Can. J. For. Res. 27, 453–463.
Hernandez-Gimenez, M.J., Lucas, M.M., de Felipe, M.R., 2002. Antioxidant defense and damage in senescing lupin nodules. Plant Physiol. Biochem. 40, 645–657.
Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611.
Hoshika, Y., Moura, B., Paoletti, E., 2018. Ozone risk assessment in three oak species as affected by soil water availability. Environ. Sci. Pollut. Res. 25, 8125–8136.
Israr, M., Sahi, S., Datta, R., Sarkar, D., 2006. Bioaccumulation and physiological effects of mercury in Sesbania drummondii. Chemosphere 65, 591–598.
Jarvis, P.G., 1976. Interpretation of variations in leaf water potential and stomatal conductance found in canopies in field. Philos. Trans. R. Soc. Lond. B 273, 593–610.
Kanofsky, J.R., Sima, P.D., 2005. Assay for singlet oxygen generation by plant leaves exposed to ozone. Methods Enzymol. 319, 512–520.
Kivimäenpää, M., Jonsson, A.M., Stjernquist, I., Sellden, G., Sutinen, S., 2004. The use of light and electron microscopy to assess the impact of ozone on Norway spruce needles. Environ. Pollut. 127, 441–453.
Kraus, T.E., Evans, R.C., Fletcher, R.A., Paul, S.K.P., 1995. Paclobutrazol enhances tolerance to increased levels of UV-B radiation in soybean (*Glycine max*) seedlings. Can. J. Bot. 73, 797–806.
Landi, M., 2017. Commentary to: "Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds". Planta 245 (6), 1097.
Levin, A.G., Pignata, M.L., 1995. *Ramalina ecklonii* as a bioindicator of atmospheric pollution in Argentina. Can. J. Bot. 73, 1196–1202.
Lodovici, M., Bigagli, E., 2011. Oxidative stress and air pollution exposure. J. Toxicol. 2011, 1–9.
Lopes, G.K.B., Schulman, H.M., Hermes-Lima, M., 1999. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Biochim. Biophys. Acta 1472, 142–152.
López, A., Montaño, A., Garcia, P., Garrido, A., 2005. Note: quantification of ascorbic acid and dehydroascorbic acid in fresh olives in commercial presentations of table olives. Food Sci. Technol. Int. 11, 199–204.
Manning, W.J., Godzik, B., 2004. Bioindicator plants for ambient ozone in Central and Eastern Europe. Environ. Pollut. 130, 33–39.
Matyssek, R., Sandermann, H., 2003. Impact of ozone on trees: an ecophysiological perspective. Prog. Bot. 64, 349–404.
Matyssek, R., Wieserb, G., Calfapietra, C., de Vriesd, W., Dizengremelf, P., Ernst, D., Jolivet, Y., Mikkelsen, T.N., Mohren, G.M.J., Le Thiec, D., Tuovinen, J.P., Weatherall, A., Paoletti, E., 2012. Forests under climate change and air pollution: gaps in understanding and future directions for research. Environ. Pollut. 160, 57–65.
Moura, B.B., Souza, S.R., Alves, E.S., 2011. Structural responses of *Ipomoea nil* (L.) Roth "Scarlet O'Hara" (Convolvulaceae) exposed to ozone. Acta Bot. Bras. 25, 122–129.
Moura, B.B., Hoshika, Y., Ribeiro, R.V., Paoletti, E., 2017. Exposure- and flux-based assessment of ozone risk to sugarcane plants. Atmos. Environ. 176, 252–260.
Moura, B.B., Alves, E.S., Marabesi, M.A., Souza, S.R., Schaub, M., Vollenweider, P., 2018. Ozone affects leaf physiology and causes injury to foliage of native tree species from the tropical Atlantic forest of southern Brazil. Sci. Total Environ. 610–611, 912–925.
Ocampo, J., d'Eeckenbrugge, G.C., Jarvis, A., 2010. Distribution of the genus *Passiflora* L. diversity in Colombia and its potential as an indicator for biodiversity management in the coffee growing zone. Diversity 2, 1158–1180.
Oksanen, E., Sober, J., Karnosky, D.F., 2001. Impacts of elevated $CO_2$ and/or $O_3$ on leaf ultrastructure of aspen (*Populus tremuloides*) and birch (*Betula papyrifera*) in the aspen FACE experiment. Environ. Pollut. 115, 437–446.

Otobone, F.J., Martins, J.V.C., Trombelli, M.A., Andreatini, R., Audi, E.A., 2005. Anxiolytic and sedative effects of a combined extract of *Passiflora alata* Dryander and *Valeriana officinalis* L. in rats. Acta Sci. Health Sci. 27, 145–150.

Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., Badea, O., 2017. A new-generation 3D ozone FACE (Free Air Controlled Exposure). Sci. Total Environ. 575, 1407–1414.

Pell, E.J., Schlagnhaufer, C.D., Arteca, R.N., 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. Physiol. Plant. 100, 264–273.

Pérez-Salicrup, D.R., Schnitzer, S.A., Putz, F.E., 2004. The community ecology and management of lianas. For. Ecol. Manag. 190, 1–2.

Pertuzatti, P.B., Sganzerla, M., Jacques, A.C., Barcia, M.T., Zambiazi, R.C., 2015. Carotenoids, tocopherols and ascorbic acid contente in yellow passion fruit (*Passiflora edulis*) grown under different cultivation systems. LTW Food Sci. Technol. 64, 259–263.

Phillips, O.L., Martinez, R.V., Arroyo, L., Baker, T.R., Killeen, T., Lewis, S.L., Malhi, Y., Mendoza, A.M., Neill, D., Vargas, P.N., Alexiades, M., Cerón, C., Di Fiore, A., Erwin, T., Jardim, A., Palacios, W., Saldias, M., Vinceti, B., 2002. Increasing dominance of large lianas in Amazonian forests. Nature 418, 770–774.

Pivello, V.R., Vieirab, M.V., Grombone-Guaratinic, M.T., Matos, D.M.S., 2018. Thinking about super-dominant populations of native species – examples from Brazil. Perspect. Ecol. Conserv. 16, 74–82.

Puckette, M.C., Weng, H., Mahalingam, R., 2007. Physiological and biochemical responses to acute ozone-induced oxidative stress in *Medicago truncatula*. Plant Physiol. Biochem. 45, 70–79.

Reddy, A.R., Chaitanya, K.V., Jutur, P.P., Sumithra, K., 2004. Differential antioxidative responses to water stress among five mulberry (*Morus alba* L.) cultivars. Environ. Exp. Bot. 52, 33–42.

Roshchina, V.V., 2008. Fluorescing World of Plant Secreting Cells. Science Publishers, Enfields.

Roshchina, V.V., Mel'nikova, E.V., 2001. Pollen chemosensitivity to ozone and peroxides. Russ. J. Plant Physiol. 48, 74–83.

Roshchina, V.V., Roshchina, V.D., 2003. Ozone and Plant Cell. Kluwer Academic Publishers, Dordrecht.

Roshchina, V.V., Yashin, V.A., Kuchin, A.V., 2015. Fluorescent analysis for bioindication of ozone on unicellular models. J. Fluoresc. 25, 595–601.

Rossell, I.M., Eggleston, H., 2017. Elevational distribution of temperate lianas along trails in Pisgah National Forest. Southeast. Nat. 16, 443–450.

Sá, A.F.L., Valeri, S.V., Cruz, M.C.P., Barbosa, J.C., Rezende, G.M., Teixeira, M.P., 2014. Effects of potassium application and soil moisture on the growth of *Corymbia citriodora* plants. Cerne 20, 645–651.

Saitanis, C.J., 2003. Background ozone monitoring and phytodetection in the greater rural area of Corinth-Greece. Chem. Aust. 51, 913–923.

Sant'Anna-Santos, B.F., Silva, L.C., Azevedo, A.A., Aguiar, R., 2006. Effects of simulated acid raind on leaf anatomy and micromorphology of *Genipa americana* L. (Rubiaceae). Braz. Arch. Biol. Technol. 49, 313–332.

Santos, A.C.R., Furlan, C.M., 2013. Levels of phenolic compounds in *Tibouchinapulchra* after fumigation with ozone. Atmos. Pollut. Res. 4, 250–256.

Scandalios, J.G., 1993. Oxygen stress and superoxide dismutases. Plant Physiol. 101, 7–12.

Scherer, C.C., 2014. Conservação filogenética de nicho climático para espécies do gênero *Passiflora* L. (Passifloraceae) com ocorrência no Brasil. Universidade Federal do Paraná, Curitiba (M.Sc. thesis).

Schnitzer, S.A., 2005. A mechanistic explanation for global patterns of liana abundance and distribution. Am. Nat. 166, 262–276.

Schnitzer, S.A., Bongers, F., 2002. The ecology of lianas and their role in forests. Trends Ecol. Evol. 17, 223–230.

Silva, L.D., Oliva, M.A., Azevedo, A.A., Araújo, J.M., Aguiar, R.M., 2005. Micromorphological and anatomical alterations caused by simulated acid rain in Restinga plants: *Eugenia uniflora* and *Clusia hilariana*. Water Air Soil Pollut. 168, 129–143.

Soja, G., Eid, M., Gangl, H., Redl, H., 1997. Ozone sensitivity of grapevine (*Vitis vinifera* L.): evidence for a memory effect in a perennial crop plant? Phyton 37, 265–270.

Souza, P.U., Lima, L.K.S., Soaresb, T.L., Jesus, O.N., Filho, M.A.C., Girardi, E.A., 2018. Biometric, physiological and anatomical responses of *Passiflora* spp. to controlled water deficit. Sci. Hortic. 229, 77–90.

Vollenweider, P., Ottiger, M., Günthardt-Goerg, M.S., 2003. Validation of leaf ozone symptoms in natural vegetation using microscopical methods. Environ. Pollut. 124, 101–118.

Vollenweider, P., Fenn, M.E., Menard, T., Günthardt-Goerg, M., Bytnerowicz, A., 2013. Structural injury underlying mottling in ponderosa pine needles exposed to ambient ozone concentrations in the San Bernardino Mountains near Los Angeles, California. Trees 27, 895–911.

Wang, A.G., Luo, G.H., 1990. Quantitative relation between the reaction of hydroxylamine and superoxide anion radicals in plants. Plant Physiol. Commun. 6, 55–57.

Wetzel, S., Demmers, C., Greenwood, J.S., 1989. Spherical organelles, analogous to seed protein bodies, fluctuate seasonally in parenchymatous cell of hardwoods. Can. J. Bot. 67, 3439–3445.

Wintermans, J.F.G.M., De Mots, A., 1965. Spectrophotometric characteristics of chlorophylls *a* and *b* and their phenophytins in ethanol. Biochim. Biophys. Acta 109 (2), 448–453.

Wohlmuth, H., Penman, K.G., Pearson, T., Lehmann, R.P., 2010. Pharmacognosy and chemo types of passionflower (*Passiflora incarnata* L.). Biol. Pharm. Bull. 33, 1015–1018.

Xu, H., Vavilin, D., Vermaas, W., 2001. Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. 98, 14168–14173.

Yudasheva, L.N., Carvalho, E.B., Castanho, M.T.J.A., Krasilnikov, O.V., 2005. Cholesterol-dependent hemolytic activity of *Passiflora quadrangularis* leaves. Braz. J. Med. Biol. Res. 38, 1061–1070.

Yuting, C., Rongliang, Z., Zhongjian, J., Yong, J., 1990. Flavonoids as superoxide scavengers and antioxidants. Free Radic. Biol. Med. 9 (1), 19–21.

**Considerações Finais**

Os resultados obtidos e apresentados nos capítulos anteriores, permitiram validar três das quatro hipóteses formuladas.

(1) Espécies arbóreas pioneiras possuem características morfo-anatômicas que restringem ou evitam os efeitos do estresse oxidativo causados por fatores de estresse naturais e antrópicos e, portanto, são mais tolerantes que as espécies arbóreas não pioneiras.

Espécies arbóreas pioneiras são mais tolerantes, que as espécies não pioneiras ao estresse oxidativo, pois produzem folhas com caracteristicas morfo-anatomicas distintas que restringem ou evitam os efeitos do estresse oxidativos por fatores naturais e/ou antrópicos.

(2) Estas características morfo-anatômicas são mais evidentes em espécies arbóreas de remanescentes florestais expostas a condições ambientais mais extremas, como o clima tropical sazonal definido por períodos secos e úmidos bem marcados e altos níveis de poluentes atmosféricos emitidos por fontes urbanas, industriais e agrícolas.

A hipótese foi testada e rejeitada. A ausência de variação espacial nas características morfo-anatômicas foliares funcionais pode sugerir a influência da história evolutiva das espécies avaliadas.

(3) Espécies com menor potencial de tolerância (espécies sensíveis) a estresses ambientais (variações e anormalidades climáticas e/ou poluentes atmosféricos) apresentam uma maior variedade de marcadores microscópicos em suas lâminas foliares aparentemente saudáveis em resposta ao estresse oxidativo do que espécies com maior potencial de tolerância.

Respostas microscópicas, apesar de disseminadas entre as espécies com diferentes graus de sensibilidade ao estresse oxidativo, foram mais evidenciadas nas espécies sensíveis. Um novo marcador microscópico foi proposto para as espécies tolerantes. Com base nos marcadores microscópicos, foi possível validar o nível de tolerância ao estresse oxidativo em cada grupo de espécies.

(4) *Passiflora edulis* tem uma alta capacidade de tolerar o estresse oxidativo causado pela exposição ao ozônio, podendo dominar áreas perturbadas da Mata Atlântica.

*Passiflora edulis* é tolerante ao $O_3$, uma vez que, na presença desse gás, desenvolveu injúrias visíveis para evitar a propagação de danos, alterações estruturais e aumento dos compostos de defesa constitutiva, e que permitiram a aclimatação das plantas ao estresse oxidativo. A alta plasticidade de resposta permite a espécie dominar áreas perturbadas da Mata Atlântica.

No presente estudo, a abordagem de diferentes grupos funcionais ajudou não só no entendimento da plasticidade de resposta das plantas à pressão oxidativa nos remanescentes de Mata Atlântica do sudeste brasileiro, mas também a esclarecer os mecanismos que governam a distribuição e a abundância de espécies em um ambiente tropical perturbado. Dentre os três grandes grupos funcionais aqui estudados: as espécies arbóreas pioneiras e lianas são tolerantes ao estresse oxidativo e, portanto, possuem maior capacidade de dominar áreas antropizadas da Mata Atlântica, enquanto as espécies não pioneiras são suscetíveis ao estresse oxidativoe apresentam baixa plasticidade de resposta a mudanças ambientais e menor capacidade de dominar áreas perturbadas.

Finalmente, esta tese ressalta a relevância das características morfo-anatomicas foliares e as respostas estruturais (referidas como marcadores microscópicos) para o

entendimento das estratégias de aclimatação/adaptação ao estresse oxidativo dos diferentes grupos funcionais de plantas, colocando-as como peças fundamentais em estudos que buscam modelos de respostas em nível ecossistêmico à estressores ambientais.