MARCELA REGINA GONÇALVES DA SILVA ENGELA

Perfil metabólico de espécies nativas de Mata Atlântica expostas a estressores ambientais

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

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À minha família...
Dedico...

“Tenho um pouco de medo: medo ainda de me entregar pois o próximo instante é o desconhecido. O próximo instante é feito por mim? ou se faz sozinho? ”

(Clarice Lispector)

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RESUMO

Os ecossistemas florestais estão naturalmente sujeitos a diversos fatores de estresse oxidativo. Devido à expansão das cidades, o aumento das atividades agrícolas e industriais, os ecossistemas vêm sendo fragmentados e afetados por fatores antrópicos de estresse, como o aumento das concentrações de poluentes gasosos e particulados. O ozônio é considerado um dos poluentes atmosféricos mais importantes em áreas urbanas e rurais, por ser altamente fitotóxico. Outro processo comum nos centros urbanos é a queima de combustíveis fósseis, que emite quantidades consideráveis de óxidos de nitrogênio (NOx), um dos precursores da formação do O_3 a partir de processos fotoquímicos na atmosfera. Somado a isso, nos últimos anos, alta deposição de compostos de nitrogênio gasosos, dissolvidos na água de chuva ou adsorvidos ao material particulado tem sido observada em ecossistemas terrestres, especialmente em regiões com alta densidade populacional e intensa atividade agrícola. Os poluentes gasosos, como o NO_2 e O_3, entram nas plantas através dos estômatos e são metabolizados em diferentes compartimentos celulares, onde decompõem-se espontaneamente em solução aquosa, formando espécies reativas de oxigênio (ERO), que podem causar a degradação de lipídeos, ácidos nucleicos, proteínas e pigmentos. Ainda, podem causar alterações na fotossíntese, ocasionando em mudanças nas características dos sinais de fluorescência das clorofilas, redução da Rubisco, redução da condutância estomática e consequentemente a redução da assimilação líquida de carbono. Entretanto, a literatura demonstra que determinados compostos podem auxiliar o sistema de defesa vegetal na eliminação das ERO, destacando-se nos últimos o papel dos carboidratos, aminoácidos, ácidos orgânicos e substâncias fenólicas. Através da metabolômica, é possível verificar as alterações que os diferentes estresses ambientais poderiam causar no metabolismo vegetal. Assim, a presente tese utilizou a abordagem ecossistêmica, sendo estudado o perfil de espécies representativas de três grupos funcionais da Floresta Atlântica (espécies arbóreas pioneiras, arbóreas não pioneiras e lianas). Para tanto, três etapas experimentais foram realizadas: (1) 21 espécies arbóreas pertencentes a diferentes grupos funcionais foram selecionadas e estudadas durante os períodos chuvoso e seco de 2016 em quatro remanescentes florestais do sudeste do brasileiro; (2) *Eugenia uniflora*, espécie não pioneira, foi exposta em 2017 ao O_3 em sistema O_3-FACE (Free-Air Controlled Exposure); (3) *Passiflora edulis*, uma liana tropical, foi exposta em 2019 ao ozônio em sistema O_3-FACE com acréscimo de N ao solo. Identificamos nas 21 espécies, por GC-EIMS, 13 carboidratos, 6 ácidos graxos, 4 ácidos orgânicos, ácido ascórbico, 3 aminoácidos, 3 ácidos fenólicos, 2 terpenos e 2 alcanos, enquanto as análises por HPLC-DAD detectaram flavonoides. As análises de componentes principais (APC) não revelaram distinções claras entre espécies pioneiras e não pioneiras, mas evidenciou uma variação sazonal no perfil metabólico dessas espécies. Ocorreu em resposta a fatores de estresse naturais e antrópicos de acordo com análises não métricas de escalonamento multidimensional, sugerindo ser uma resposta de aclimatação a esses múltiplos estresses ambientais. *Eugenia uniflora* mostrou-se sensível ao estresse oxidativo causado pelo ozônio, mostrando injúrias clássicas induzidas por O_3, entre as quais: redução nas concentrações de carboidratos e ácidos graxos, mudanças não significativas no perfil dos polifenóis, respostas antioxidantes ineficientes, conteúdo aumentado de ERO e indicadores de peroxidação lipídica, reduções na condutância estomática, fotossíntese líquida, razão raiz/parte aérea e crescimento em altura. No entanto, foram também observados alguns mecanismos de compensação, como o aumento da concentração foliar de polióis, representando uma para proteção às membranas e aumento do número de folhas para compensar o declínio da taxa fotossintética. Por fim, o último experimento

conduzido com *Passiflora edulis* expostas ao O_3 e a adição de nitrogênio no solo foi possível verificar que o suprimento de N amenizou os efeitos dos níveis elevados de O_3 sobre o crescimento, biomassa, fotossíntese, fluorescência da clorofila e elevou os componentes de defesa antioxidante analisados em *P. edulis*. A ausência de dano foliar visível, não redução no crescimento, produção de biomassa e aumentos significativos nos ácidos graxos, polióis, aminoácidos (como prolina), ácido ascórbico, concentrações de flavonóides e o aumento no número de folhas sugerem que a cultivar estudada é capaz de tolerar o estresse oxidativo induzido pelos efeitos interativos do O_3 e da adição de nitrogênio no solo. Os resultados obtidos na tese, ajudam a preencher as lacunas de conhecimento sobre as respostas de espécies tropicais de diferentes grupos funcionais aos diferentes estressores ambientais em que estão diariamente expostos, principalmente ao ozônio e o efeito combinado com a adição de nitrogênio no solo.

Palavras-chave: ecossistemas, estresse oxidativo, estressores ambientais, espécies tropicais, metabólitos, ozônio, nitrogênio.

ABSTRACT

Forest ecosystems are naturally subject to several oxidative stress factors and due to the expansion of cities, the increase in agricultural and industrial activities, ecosystems have been fragmented and affected by anthropic stress factors, such as increased concentrations of gaseous and air pollution. Pollutants from natural and man-made sources, such as ozone and nitrogen dioxide, are released into the atmosphere. Ozone is considered the most important air pollutants in urban and rural areas, as it is highly phytotoxic. Another common process in urban centers is the burning of fossil fuels, which emits considerable amounts of nitrogen oxides (NOx), one of the precursors of the formation of ozone from photochemical processes in the atmosphere. In addition, in recent years, high deposition of gaseous nitrogen compounds, dissolved in rainwater or adsorbed to particulate matter has been observed in terrestrial ecosystems, especially in regions with high population density and intense agricultural activity. Gaseous pollutants, such as NO_2 and O_3, enter plants through stomata and are metabolized in different cell compartments, where they spontaneously decompose in aqueous solution, forming reactive oxygen species (ROS), which can cause the degradation of lipids, nucleic acids, proteins and pigments. Still, they can cause alterations in photosynthesis, changes in the characteristics of the fluorescence signals of chlorophylls, reduction of Rubisco, reduction in stomatal conductance and consequently the reduction in net carbon assimilation. As a consequence of these changes, there may be a decrease in metabolites, especially those directly linked to photosynthesis. However, the literature demonstrates that certain compounds can assist the defense system of plants in the elimination of ROS, especially in the latter the role of carbohydrates, amino acids, organic acids and phenolic substances. Through metabolomics, it is possible to verify the changes that environmental stress, especially pollutants could cause in plants. Thus, the present thesis uses the ecosystem approach, where biochemical, physiological leaf characteristics of species representative of three functional groups of the Atlantic Forest (pioneer tree species, non-pioneer trees and lianas) were studied. For this, three experimental steps were carried out: (1) 21 tree species were selected and studied during the rainy and dry periods of 2016 in four forest remnants in southeastern Brazil; (2) *Eugenia uniflora*, a non-pioneer species, was subjected to O_3 in 2017 in the O_3-FACE system (Free-Air Controlled Exposure); (3) *Passiflora edulis*, a species of liana, was subjected to O_3 in 2019 in O_3-FACE (Free-Air Controlled Exposure) system with addition of N to the soil. We identified in the 21 species of the different functional groups, through the analysis in GC-EIMS, 13 carbohydrates, 6 fatty acids, 4 organic acids, ascorbic acid, 3 amino acids, 3 phenolic acids, 2 terpenes and 2 alkanes and the HPLC-DAD analyzes detected 5 flavonoids. Principal component analyzes (PCA) did not reveal clear distinctions between pioneer and non-pioneer species and also showed a seasonal variation in the metabolic profile of tree species. It occurred in response to natural and anthropic stressors according to non-metric multidimensional scaling analyzes and appeared to be an acclimatization response to these multiple environmental stresses. However, *Eugenia uniflora*, was sensitive to oxidative stress caused by ozone, since we found a classic O_3 lesion: reduced carbohydrate and fatty acid concentrations, non-significant changes in the polyphenols profile, inefficient antioxidant responses, increased ROS content and indicators of lipid peroxidation, reductions in stomatal conductance, liquid photosynthesis, root / shoot ratio and height growth. However, we also found some compensation mechanisms, such as increasing the leaf concentration of polyols to protect the membranes and increasing the number of leaves to compensate for the decline in the photosynthetic rate. Finally, the last experiment conducted with *Passiflora edulis* exposed to ozone and the addition of nitrogen to the

soil, it was possible to verify that the supply of N mitigated the effects of high levels of O_3 on growth, biomass, photosynthesis, chlorophyll fluorescence and increased antioxidant defense components analyzed in *P. edulis*. The absence of visible leaf damage, no reduction in growth, biomass production and significant increases in fatty acids, polyols, amino acids (such as proline), ascorbic acid, flavonoid concentrations and the increase in the number of leaves suggest that the studied cultivar is capable to tolerate oxidative stress induced by the interactive effects of O_3 and the addition of nitrogen in the soil. The results obtained in the thesis, help to fill the knowledge gaps on the responses of tropical species of different functional groups to the different environmental stressors in which they are exposed daily, mainly to ozone and the combined effect with the addition of nitrogen in the soil.

Keywords: ecosystems, oxidative stress, environmental stressors, tropical species, metabolites, ozone, nitrogen.

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1. Introdução geral

Os ecossistemas florestais estão naturalmente sujeitos a diversos fatores de estresse oxidativo causados por oscilações climáticas, quando expostos a perturbações ambientais na região onde se encontram, como, por exemplo, extremos de temperatura, déficit hídrico e excesso de radiação solar (Allen *et al*., 2015; Häder & Barnes, 2019). Além disso, devido à expansão das cidades e das atividades agrícolas e industriais, tais ecossistemas vêm sendo fragmentados e afetados por fatores antrópicos de estresse, como o aumento das concentrações de poluentes gasosos e particulados (Luo *et al*., 2019; Sanaullah *et al*., 2020; Takahashi *et al.*, 2020).

A troposfera é a camada da atmosfera onde são lançados os poluentes provenientes de fontes naturais e antrópicas (Sharma *et al*., 2017; Blondet *et al*., 2019). Durante as últimas décadas, as concentrações de poluentes atmosféricos têm aumentando consideravelmente, principalmente devido ao desenvolvimento crescente das atividades humanas, ao crescimento econômico, ao intenso tráfego de veículos e ao aumento das atividades industriais (Cetesb, 2018; Hulkkonen *et al*., 2019).

Os poluentes são classificados como primários quando são diretamente lançados na atmosfera como óxidos de nitrogênio (NOx), dióxido de enxofre e hidrocarbonetos. Quando provenientes de reações atmosféricas entre poluentes primários ou outras substâncias naturais, mediadas por variáveis meteorológicas como, temperatura e radiação solar são classificados como secundários (Laumbach *et al*., 2014; IARC, 2016; Cetesb, 2018).

O ozônio, em particular, é considerado um dos poluentes atmosféricos mais importantes em áreas urbanas e rurais, por ser oxidante e altamente fitotóxico. Um processo muito comum em grandes centros urbanos, a queima de combustíveis fósseis, emite quantidades consideráveis de poluentes gasosos, como NOx, precursores da formação do ozônio a partir de processos fotoquímicos na atmosfera (Sandrin *et al*., 2018; Lin *et al*., 2019; Pinto *et al*., 2019). Ainda, nos últimos anos, alta deposição de compostos de nitrogênio gasosos, dissolvidos na água de chuva ou adsorvidos ao material particulado tem sido observada em ecossistemas terrestres, especialmente em regiões com alta densidade populacional e intensa atividade agrícola (Kanakidou *et al*., 2016; Ellermann *et al*., 2018).

Os poluentes gasosos, como o dióxido de nitrogênio e ozônio, entram no corpo vegetal através dos estômatos e são metabolizados em diferentes compartimentos celulares,

como cloroplastos, mitocôndrias e peroxissomos. No apoplasto, esses poluentes gasosos decompõem-se espontaneamente em solução aquosa, formando espécies reativas de oxigênio (ERO). As ERO compreendem formas livres radicalares, como radical superóxido ($O_2^{\cdot -}$), radical hidroxila ($\cdot OH$), radical peridroxila ($HO_2\cdot$) e radical alcoxila ($RO\cdot$), e formas não radicais como o peróxido de hidrogênio (H_2O_2) e o oxigênio singleto ($^1O_2$), mas todas, em excesso, podem ser prejudiciais para as células vegetais. As ERO podem causar efeitos fisiológicos, bioquímicos e alterações estruturais, pois atacam rapidamente moléculas vitais, como lipídeos, ácidos nucleicos, proteínas, pigmentos e outros componentes dentro e fora das células vegetais, ocasionando até mesmo a morte celular, dependendo de suas concentrações no citoplasma (Halliwel & Gutteridge, 2007; Avery, 2014; Shi *et al*., 2017).

Os danos às membranas podem ser um dos primeiros sinais de estresse proporcionado pelo aumento dos níveis de ERO, podendo ocorrer um aumento da permeabilidade das membranas e consequente liberação de eletrólitos (Alonso *et al*., 1997). Ainda, podem ocorrer alterações estruturais, como por exemplo, o espessamento da parede celular, surgimento de protrusões no mesofilo e obstrução do espaço intercelular (Paoletti *et al*., 2009; Moura *et al*., 2018).

Sabe-se que o processo de fotossíntese pode também ser afetado pelos produtos formados a partir da absorção dos poluentes, em consequência de alterações no estado funcional das membranas dos tilacoides dos cloroplastos. A fixação limitada de CO_2 devido ao estresse leva a uma redução de carbono pelo Ciclo de Calvin e diminuição do $NADP^+$ oxidado. Este último serve como aceptor de elétrons na fotossíntese quando a ferredoxina (Fd) é reduzida em excesso durante a transferência de elétrons, podendo ser transferido do fotossistema I (PSI) para O_2 e formar $\cdot O_2^-$ pelo processo chamado reação de Mehler (Thirupathi *et al*., 2011). Ainda, os poluentes podem promover mudanças nas características dos sinais de fluorescência de clorofilas. Em resposta a alterações no transporte de elétrons, ocorre a redução da atividade da enzima Ribulose-1,5-bisfosfato carboxilase oxigenase (Rubisco) e redução da condutância estomática (Gerosa *et al*., 2009; Calatayud *et al*., 2010; Feng *et al*., 2011, Tao *et al*., 2018), resultando na redução da assimilação líquida de carbono.

Um melhor entendimento dos mecanismos genéticos, fisiológicos e bioquímicos que atuam na proteção das plantas frente aos diferentes estresses ambientais vem sendo apoiado pelos avanços em abordagens "ômicas", como genômica, proteômica, metabolômica e interactômica (Shen *et al*., 2018; Gautam *et al*., 2020; Li *et al*., 2020).

Abordagens proteômica e metabolômica podem nortear o entendimento da composição e quantidades de proteínas e metabólitos (Li *et al*., 2019). Segundo Fiehn (2002), estes são os produtos intermediários ou finais do metabolismo e que desempenham importante papel em diversos processos fisiológicos e de desenvolvimento vegetal (Dai *et al*., 2019; Gonulalan *et al*., 2019).

Metabolismo, assim, pode ser definido como o conjunto de reações químicas que ocorre constantemente nas células de qualquer organismo vivo. Didaticamente, o metabolismo pode ser dividido em primário e secundário. O metabolismo primário vegetal é o conjunto de reações responsável pela síntese de compostos essenciais para produção de energia e constituição do protoplasto. A ele estão associados todos os processos que sintetizam a formação de ácidos carboxílicos, aminoácidos, carboidratos, ácido graxos, proteínas e ácidos nucléicos. Sabe-se que a fotossíntese é um processo vital, a partir do qual os vegetais convertem energia luminosa, dióxido de carbono, água e íons inorgânicos em compostos orgânicos essenciais para manutenção e sobrevivência. Os compostos orgânicos resultantes são então utilizados pelas plantas para a síntese de compostos intermediários, que originarão os compostos do metabolismo secundário.

Entende-se por metabolismo vegetal secundário o conjunto de reações que leva à síntese de compostos envolvidos na defesa e na comunicação das plantas com o ambiente em seu entorno (Groenigen *et al*., 2015). Metabólitos secundários em organismos fotossintetizantes são produzidos a partir de quatro vias de síntese: via do ácido chiquímico (ou chiquimato), via do ácido malônico (ou malonato), via do ácido mevalônico (ou mevalonato) e via do metileritritol fosfato (Dewick, 2002; Gárcia & Carril, 2009). Essas vias estão associadas à síntese de três grandes grupos de metabolitos secundários: substâncias nitrogenadas (como alcaloides, glucosinolatos e glicosídeos cianogênicos), terpenos e esteroides, e substâncias fenólicas (como flavonoides e taninos, por exemplo).

Dentre os metabólitos secundários, destacam-se as substâncias fenólicas, caracterizadas por possuírem um anel aromático com pelo menos um grupo hidroxila (Cartea *et al*., 2011), constituindo um vasto número de substâncias já descritas e classificadas em diferentes subgrupos: ácidos fenólicos, fenilpropanoides, flavonoides, cumarinas, taninos, lignoides e ligninas (Haminiuk *et al*., 2012).

Os metabólitos secundários estão relacionados à ação protetora natural de plantas aos diferentes estresses bióticos (ação de herbívoros e patógenos, por exemplo) e abióticos, como aqueles associados às mudanças de temperatura, conteúdo de água, níveis

de luz, radiação UV, níveis de poluentes e deficiência de nutrientes minerais (Bieski *et al*, 2004; Gobbo-Neto *et al*, 2007; Moraes *et al*., 2011; Kliebenstein, 2012). Aos flavonoides também é associada a função fotoprotetora contra os raios ultravioleta que incidem principalmente sobre as folhas. As substâncias fenólicas e terpênicas podem minimizar, ainda, os danos causados pelo aumento da formação de ERO em plantas expostas a poluentes (Simões *et al*, 2010; Agati *et al*., 2012; Agati *et al*., 2013).

Visto que a síntese de metabólitos secundários está relacionada com a síntese de metabólitos primários, alguns estudos têm demonstrado que a ação de poluentes oxidantes na fotossíntese e nos teores de açúcares e aminoácidos afeta, por consequência, a produção de substâncias fenólicas, podendo alterar a capacidade de defesa das plantas a outros estresses (Pasqualini *et al*., 2003; Furlan *et al*., 2004; Tripathi & Gautam, 2006).

Em consequência de eventos múltiplos de oxidação proporcionados pela formação de ERO induzida por estressores ambientais, os danos metabólicos em células vegetais, como os descritos anteriormente, podem se projetar para níveis mais altos da organização biológica. Sabe-se que além dos danos imediatos como necroses foliares, os estressores ambientais podem causar reduções no crescimento, produtividade, alocação de recursos para as raízes, produção de sementes, podendo afetar a dinâmica de populações (Deepak & Agrawal, 2001, Ashmore, 2005; Bender *et al*., 2006; Wang *et al*., 2012; Emberson *et al*., 2018; Loka *et al*., 2018), eliminando espécies mais sensíveis e favorecendo o domínio de espécies mais resistentes, resultando no declínio de florestas, como já observado nos Estados Unidos e Europa (Arndt *et al*., 1995; Percy, 2003).

No Brasil, há um déficit de estudos sobre os efeitos oxidativos associados aos estressores ambientais em florestas ou sobre o metabolismo das espécies vegetais nativas que possam indicar sua capacidade de se aclimatar aos mesmos. São raros também aqueles que relacionam poluentes aéreos com metabólitos secundários em plantas. Destacam-se, por exemplo, os trabalhos de Furlan *et al*. (1999), que verificaram maiores conteúdos de nitrogênio e menores concentrações de compostos fenólicos e taninos totais em plantas de *Pleroma raddianum* (Manacá-da-Serra, anteriormente denominada *Tibouchina pulchra*), uma espécie pioneira nativa da Mata Atlântica, nas proximidades do polo industrial de Cubatão, em São Paulo e o de Domingos *et al*. (2015), que verificaram as diferenças nas concentrações de carboidratos, taninos, compostos fenólicos e o *status* do sistema antioxidante enzimático e não enzimático em *Piptadenia gonoacantha*, *Croton floribundus* e *Astronium graveolens*, espécies nativas de fragmentos de Mata Atlântica sob diferentes níveis de poluição atmosférica.

No entanto, ao se estudar os impactos de estresses ambientais em florestas tropicais e subtropicais, deve-se considerar a alta biodiversidade que estas apresentam. Essa característica dificulta a reprodução de modelos metodológicos desenvolvidos para ambientes de clima temperado, onde é possível avaliar e inferir sobre os efeitos oxidativos causados por estresses ambientais às florestas com base em características morfológicas, fisiológicas e metabólicas de poucas espécies. Uma alternativa é fazer o uso de uma abordagem ecossistêmica funcional nas florestas com alta biodiversidade. Tal abordagem consiste em analisar as mesmas características em espécies com funções ecológicas distintas e que sejam representativas de seu grupo funcional. A escolha das espécies pode ser baseada, por exemplo, no estágio sucessional ao qual pertencem.

Bussotti (2008), em artigo de revisão sobre características foliares funcionais em espécies arbóreas nativas de ecossistemas florestais na região do Mediterrâneo, concluiu que as espécies de estágios sucessionais iniciais apresentam menor tolerância ao estresse oxidativo do que as espécies secundárias tardias, devido a características foliares estruturais e funcionais. Estas espécies com maior resiliência ao estresse apresentam, por exemplo, menor concentração de N nas folhas, maior síntese de compostos de defesa, abundância de estruturas mecânicas foliares e alto teor de clorofilas, entretanto, o contrário ocorre em plantas com menor resiliência ao estresse.

Por outro lado, estudos recentes parecem indicar que as respostas adaptativas de espécies arbóreas nativas da Floresta Atlântica ao estresse oxidativo são opostas às sintetizadas por Bussotti (2008) em espécies nativas das florestas mediterrâneas, destacando a necessidade de aprofundar o conhecimento acerca do tema nas regiões tropicais.

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 tolerância ao sombreamento: as intolerantes ao sombreamento (espécies pioneiras) e as tolerantes ao sombreamento (espécies secundárias tardias). As espécies pioneiras têm sido caracterizadas por taxas mais altas de fotossíntese e de acúmulo de biomassa e menor conteúdo de pigmentos do que as espécies secundárias tardias. Esses autores acrescentaram, ainda, que o impacto da alta radiação sobre o desempenho fisiológico foi notadamente mais intenso nas espécies secundárias tardias. Aguiar *et al*. (2016) avaliaram pela primeira vez respostas antioxidantes associadas ao ciclo ascorbato-glutationa em folhas de uma espécie pioneira (*Croton floribundus*), uma secundária inicial (*Piptadenia gonoacantha*) e uma secundária tardia (*Astronium*

graveolens) ocorrentes em fragmentos de Floresta Atlântica Semidecidual, na região metropolitana de Campinas, como indicadoras do aumento da tolerância ao estresse oxidativo induzido por poluentes atmosféricos e clima tropical sazonal. A espécie pioneira mostrou ser a espécie mais eficiente em termos de tolerância ao estresse oxidativo quando comparada às espécies não pioneiras. Engela (2016) observou, em amostragens paralelas às realizadas por Aguiar *et al.* (2016), que carboidratos (principalmente amido) e substâncias fenólicas (principalmente flavonoides) foram marcadores adequados do aumento de tolerância aos estresses ambientais em folhas de *C. floribundus* e *A. graveolens*, respectivamente. Os níveis foliares desses compostos tenderam a aumentar em resposta aos aumentos de radiação solar, umidade relativa e temperatura do ar e a diminuir em resposta ao aumento da concentração de ozônio e dióxido de nitrogênio.

Brandão *et al*. (2017) e Esposito *et al*. (2018), ao descreverem o potencial antioxidante de árvores adultas pioneiras e não pioneiras representativas de remanescentes de Floresta Atlântica no Estado de São Paulo e Minas Gerais, verificaram que as espécies pioneiras tenderam a ser mais tolerantes ao estresse oxidativo, sendo a formação de ERO mais intensa nas folhas das espécies arbóreas não pioneiras. Os autores inferiram que as características bioquímicas das espécies de ambos os grupos funcionais variaram aparentemente em função de efeitos combinados de estressores ambientais.

Fernandes *et al*. (2019) verificaram que *Passiflora edulis*, uma espécie de liana encontrada facilmente em áreas perturbadas, sob condições ambientais adversas como diferentes níveis de poluição, é tolerante à exposição ao ozônio. Em estudo realizado em sistema FACE, os autores demonstraram que altas concentrações de ozônio não afetaram a fotossíntese, o conteúdo de carboidratos ou a produção de biomassa total de plantas de *P. edulis*, que também apresentaram maiores teores foliares de ácido ascórbico, glutationa, carotenoides e flavonoides, indicando assim eficientes mecanismos de tolerância.

1.1. Objetivos Gerais e Hipóteses

Este estudo visou fornecer conhecimento, em condições naturais e experimentais, sobre as variações de metabólitos (açúcares, aminoácidos, ácidos orgânicos, ácidos graxos e substâncias fenólicas) em espécies representativas de diferentes grupos funcionais (árvores pioneiras, não pioneiras e lianas) da Floresta Atlântica do sudeste brasileiro, que está sob forte influência antrópica devido à fragmentação, à poluição atmosférica e a anormalidades climáticas advindas das mudanças do uso da terra.
Assim, apoiado na base conceitual apresentada, as seguintes hipóteses foram formuladas:

H1: As espécies arbóreas pioneiras e lianas são menos susceptíveis ao estresse oxidativo induzido por fatores de estresse ambiental de origem natural e antrópica do que as espécies não pioneiras, considerando que as primeiras estão adaptadas à neutralização de níveis basais mais altos de ERO decorrentes de processos fisiológicos naturais, como fotossíntese e respiração.

H2: O perfil de metabólitos difere entre as espécies representativas dos diferentes grupos funcionais e alvos desta pesquisa (árvores pioneiras, não pioneiras e lianas), em função de seus diferentes perfis pro-oxidantes/antioxidantes.

H3: Maior diversidade metabólica ou níveis mais altos de alguns grupos de metabólitos podem incrementar a tolerância das espécies nativas frente aos fatores de estresse ambiental.

1.2. Organização da Tese

Os resultados obtidos estão apresentados em três diferentes capítulos, escritos em inglês e formatados de acordo com as normas do periódico para o qual foram ou serão submetidos:

Capítulo 1: Metabolic variations on native Atlantic Forest species: are they traits of successional stage or acclimation responses to multiple environmental stresses?

Nesse capítulo, é apresentada a abordagem do uso da metabolômica para indicar o potencial de tolerância ou sensibilidade de 21 espécies arbóreas nativas, pertencentes a dois grupos funcionais distintos, coletadas em quatro fragmentos de Mata Atlântica do sudeste brasileiro.

Capítulo 2: Metabolic and physiological alterations indicate that the tropical broadleaf tree *Eugenia uniflora* L. is sensitive to ozone.

O segundo capítulo trata da sensibilidade de uma espécie arbórea não pioneira sob o estresse oxidativo provocado pelo ozônio troposférico (O_3), em sistema FACE (Free-Air Controlled Exposure Ozone System).

Capítulo 3: **How does a tropical liana species respond to nitrogen deposition and ozone? A physiological, biochemical and metabolic approach.**

O terceiro capítulo trata das respostas fisiológicas, bioquímicas e padrões de crescimento de uma espécie de liana sob o estresse oxidativo provocado pelo ozônio troposférico (O_3) e adição de nitrogênio no solo, em sistema FACE (Free-Air Controlled Exposure Ozone System).

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Chapter 1: Metabolic variations on native Atlantic Forest species: are they traits of successional stage or acclimation responses to multiple environmental stresses? [1]

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Abstract

Forest ecosystems are subject to natural or anthropic environmental stressors, which can cause an increase in reactive oxygen species (ROS) in native plant species. ROS will initiate multiple oxidation events, causing damage, from the cellular to the ecosystem level. The intensity of these damages depends on how efficient the mobilization of antioxidant defenses is in maintaining the pro-oxidant/antioxidant balance. In this way, plants can raise concentrations of antioxidant compounds, including primary and secondary metabolites. Changes in the content and chemical composition of compounds that indicate oxidative stress are used as means of assessing the species tolerance potential to stress imposed by the environment. A field study was conducted aiming the analysis of composition and contents of primary and secondary metabolites in pioneer and non-pioneer tree species of four Atlantic Forest remnants exposed to natural and anthropic sources of oxidative stress. We raised the hypothesis that pioneer tree species have a more diverse metabolite profile, a higher capacity to change it in response to natural or anthropic environmental stresses, and a higher ability to tolerate the oxidative stress than non-pioneer species. A total of 21 tree species were included in the study. The leaf samples were collected during the rainy (January and February) and dry (June to August) periods in 2016. GC–EIMS analyses revealed 13 carbohydrates, 6 fatty acids, 4 organic acids, ascorbic acid, 3 amino acids, 3 phenolic acids, 2 terpenes, and 2 alkanes. HPLC-DAD analyses detected flavonoids. Principal component analyses (PCA) revealed no clear distinctions between pioneer and non-pioneer species, contradicting our hypothesis.

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PCA also evidenced a seasonal variation in the metabolic profile of the tree species. It occurred in response to both natural and anthropic stress factors according to non-metric multidimensional scaling analyses, suggesting to be acclimation responses of these species to the multiple environmental stresses.

Keywords: Forest ecosystems, natural stressors, reactive oxygen species, primary metabolites, flavonoids.

1. Introduction

Forests offer many ecosystem services to human populations, such as biological diversity protection, water quality and supply, flood control, carbon sequestration, climate adjustment, and recreation (Huang *et al*., 2016; Chazdon *et al*, 2017; Mello *et al*., 2018; De Freitas *et al*., 2019; Li *et al*., 2019; Gardon *et al*., 2020). However, they are naturally subject to several oxidative stress factors caused by environmental fluctuations in their natural habitats, such as temperature extremes, water deficit and excessive solar radiation. Deforestation, forest degradation, and increase of air pollution are additional environmental disturbances caused by human activities to these ecosystems (Tauz *et al*., 2007; Delgado-Aguilar *et al*., 2019; Muttaqin *et al*., 2019). All these disturbances may compromise the quality of the mentioned ecosystem services.

The troposphere is the layer of the atmosphere where pollutants from natural and anthropic sources are released, such as nitrogen oxides (NO_x), and where ozone (O_3) is formed. These gaseous pollutants penetrate the plant leaves predominantly through stomata. When in the apoplast, they are solubilized in water, inducing the formation of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), which are strong oxidants (Krupa and Manning, 1988; Brasseur and Schimel, 1999; Florentina & Ion, 2011; Silva *et al*., 2019; Wang *et al*, 2019).

ROS in excess may oxidize cellular molecules, such as lipids, nucleic acids and proteins, reducing enzyme activities. They can also interfere on the stomatal opening, decreasing intercellular CO_2 due to stomatal closure, increase the energy dissipation, reducing the photosynthesis, and consequently reduction on metabolite concentrations, such as carbohydrates, amino acids, carboxylic acids, fatty acids, and proteins. Furthermore, the oxidative stress can decrease photopigment contents and plant biomass and even cause cell death (Foyer & Noctor 2005; Halliwel & Gutteridge, 2007; Kapoor *et al*, 2019; Romero-Puertas *et al*, 2019; Han *et al*, 2020). The oxidative stress and negative effects on plant metabolism and growth caused by excessive ROS can be avoided or minimized by efficient detoxification mechanisms (Almeida *et al*, 2004; Baier *et al*, 2005; Mukherjee *et al*., 2019; Aguiar *et al*., 2015; Brandão *et al*., 2017, Esposito et al., 2018).

Non-enzymatic metabolites, such as phenolic compounds (*eg*. anthocyanins, tannins, and other flavonoids) are important antioxidants, while carbohydrates (*eg*. sucrose, glucose, fructans) are important substances inducing ascorbate and glutathione synthesis and acting as signaling molecules, they also support osmotic potential of the

cells and participate in redox reactions and have been also reported as indicators of air pollution stress (Coueé *et al*., 2006; Santos & Furlan, 2013; Krasavina *et al*., 2014; Domingos *et al*., 2015; Azzazy, 2020). However, there is still a lack of knowledge about possible correlations between the metabolic profiles of different functional groups of species living in disturbed remnants of Atlantic forest and natural or anthropic environmental stressors effects.

The negative effects of pollutants can be intensified by other environmental factors, as meteorological and physiographic features, as well as by uncontrolled industrialization, population growth, increase in motor vehicles, and agricultural activities (Rangel *et al*, 2018; Guo *et al*, 2019; Barglagi *et al*, 2019). The intensity of pollutant effects will also depend on the complexity of the plant community structure (Aguiar *et al*., 2015; Brandão *et al*., 2017; Esposito *et al*., 2018; Geng *et al*., 2015; Jiang *et al*., 2015; Tee *et al*., 2020). In this sense, forest fragments near to urban areas are under more frequent stress and can be more affected than large forest massifs (Halliwel & Gutteridge, 2007; Domingos *et al*., 2015; Bouchard *et al*., 2019; Safari & Sohrabi, 2019; Dafré-Martinelli *et al*., 2020). They are generally secondary forest formations and are composed by pioneer and non-pioneer tree species, which differ from each other by the requirement of light and tolerance to shade (Favaretto *et al*., 2011). Basically, pioneer species are intolerant to shade and non-pioneer species are tolerant to shade. These authors added that the impact of high radiation on physiological performance was notably more intense on late secondary species than on pioneer species. In addition, pioneer species have been characterized by higher rates of photosynthesis, biomass accumulation, rapid growth, and lower pigment content than late secondary species (Lorenzi, 1992; Chazdon *et al*., 1996; Nogueira *et al*., 2004; Silvestrini & Santos, 2015).

However, a more precise distinction between successional classes and their capacity to tolerate oxidative stress should be based on physiological and metabolic characteristics that allow the species to occupy or not a given environment (Lüttge & Scarano, 2007; Ribeiro *et al*., 2009; Brandão *et al*., 2017; Esposito *et al*., 2018; Fan *et al*, 2019; Arkorful *et al*, 2019). Metabolomic analysis is a good alternative to this purpose. It describes many metabolites synthesized from different chemical routes and determine their concentrations in different plant species (Fiehn, 2002; Takemoto & Arita, 2009), which in turn can be correlated with biotic and abiotic factors.

Focusing on metabolic features, we conducted this field study with the aims of: 1) determining the composition and concentrations of primary and secondary metabolites in

pioneer and non-pioneer tree species of Atlantic Forest remnants exposed to natural and anthropic sources of oxidative stress; 2) discussing if the metabolic variations are natural traits of their successional stage or acclimation responses induced by multiple anthropic stresses. Based on the knowledge available, we raised the hypothesis that pioneer tree species have a more diverse metabolite profile, a higher capacity to change it in response to natural or anthropic environmental stresses, such as temperature extremes, high radiation, and concentrations of pollutants, and a higher ability to tolerate the oxidative stress than non-pioneer species.

2. Material and methods

2.1. Sampling design

The field study was developed in four remnants of Atlantic forest located in the vicinity of different air pollution sources and exposed to natural distinct climatic conditions. Three of them are located in São Paulo State (SP) and one in Minas Gerais State (MG). They are included in the following conservation unities: Natural and Municipal Park Nascentes de Paranapiacaba (PP, Santo André/SP); State Park Fontes do Ipiranga (PEFI, São Paulo/SP); Ecological Area of Relevant Interest Mata de Santa Genebra (MSG, Campinas/SP); State Park of Itacolomi (PEIT, Ouro Preto/MG). The main characteristics of the study locations are reported in Table 1.1 More details about the sampling areas can be found in Brandão *et al*. (2017), Esposito *et al*. (2018), and Nakazato *et al*. (2020).

Three pioneer species and three non-pioneer species were selected in PP, PEFI, and MSG and two pioneer species and four non-pioneer species in PEIT (Table 1.2); the selected species belong to distinct taxonomic groups. They were previously indicated as the most abundant native tree species in floristic surveys conducted in the same forest remnants (Gomes *et al*., 2002; Lima *et al*., 2011; Pedreira & de Sousa, 2011; Domingos *et al*., 2015). The leaf samples were collected from five trees of each species in all areas during the rainy (January and February) and dry (June to August) periods in 2016. The sampling procedures were replicated in three consecutive days per seasonal period and location. The leaf collection started 100 m from the forest edge in each location and the distance between the trees was at least 100 m. For each species, leaves were combined into one sample, thus obtaining a total of three composite samples in each day per species.

The composite leaf samples were frozen in liquid nitrogen immediately after collection and stored in freezer under -80°C for further analyses.

Table 1.1 Main characteristics of the Conservation Unities included in this study.

Characteristics	Sites	
	PP	**PEFI**
City	Santo André	São Paulo
Coordinates	23°46′41″S 46°18′16″W	23°40′18″S 46°38′00″W
Mean altitude	890 m	770 m
Area	400 ha	540 ha
Soil classification	Red yellow Latosol/Argisol	Red yellow Latosol
Climate (Koeppen classification)	Cfb	Cwb
Mean annual rainfall	3300 mm	1500 mm
Predominant sources of pollutants	Chemical and steel industries	Urban pollution
	MSG	**PEIT**
City	Campinas	Ouro Preto
Coordinates	22°49′22.65″S 47°06′17.38″W	43°32′30″S 43°22′30″W
Mean altitude	670 m	1772 m
Area	252 ha	7500 ha
Soil classification	Red Yellow Latosol	Red Yellow Latosol
Climate (Koeppen classification)	Cwa	Cwb
Mean annual rainfall	1400 mm	1800 mm
Predominant sources of pollutants	Urban, agricultural and industrial	Mining activities and aluminum smelter

Source of information: PP (Paranapiacaba): Sugyama *et al*. (2009), Lima *et al*. (2011); PEFI (Ipiranga): Domingos *et a*l. (2003), Tanus *et al*. (2012); MSG (Mata de Santa Genebra): Alvares *et al*. (2014), Lopes *et al*. (2015); PEIT (Itacolomi): Werneck *et al*. (2000), Pedreira & Souza (2011).

Table 1.2. Trees species sampled in PP (Paranapiacaba), PEFI (Ipiranga), MSG (Mata de Santa Genebra) and PEIT (Itacolomi). P = pioneer tree species; NP = non-pioneer tree species.

Family/ Species	Sucessional Group	Sampling Site
Anacardiaceae		
1. Astronium graveolens Jarcq.	NP	MSG
Asteraceae		
2. *Eremanthus erythropappus* (DC.) McLeisch	P	PEIT
Euphorbiaceae		
3. *Alchornea sidifolia* Müll. Arg	P	PEFI
4. *Alchornea triplinerva* Müll. Arg	P	MSG
5. *Croton floribundus* Spreng.	P	MSG
Fabaceae		
6. *Machaerium villosum* Vogel	NP	PEIT
7. *Piptadenia gonoacantha* (Mart) J.F Macbr.	P	MSG
Lauraceae		
8. *Ocotea beulahiae* J.B. Baitello	NP	MSG
9. *Ocotea paranapiacabensis* Coe-Teixeira	NP	PP
Melastomataceae		
10. *Miconia cabucu* Hoehne	P	PEFI, PEIT, PP
11. *Pleroma raddianum* (DC.)	P	PP
Meliaceae		
12. *Guarea macrophylla* Vahl.	NP	PEFI, PP
13. *Guarea kuntiana* A. Juss.	NP	MSG
Myrsinaceae		
14. *Myrsine umbellata* Mart.	P	PP
Myrtaceae		
15. *Amaioua intermedia* Mart. ex Schult & Schult.	NP	PEFI
16. *Eugenia excelsa* O. Berg	NP	PEFI
17. *Eugenia cerasiflora* Miq.	NP	PEIT
Rubiaceae		
18. *Psychotria suterella* Müll. Arg	NP	PP
19. *Psychotria vellosiana* Benth.	NP	PEIT
Solanaceae		
20. *Solanum granulosoleprosum* Dunal	P	PEFI
Winteraceae		
21. *Drimys brasiliensis* Miers	NP	PEIT

The meteorological parameters and gaseous pollutants (O_3 and NO_2) were monitored nearby each forest remnant on a weekly basis throughout 2016. The concentrations of O_3 and NO_2 were measured using passive samplers installed 2 m above ground. O_3 was sampled in impregnated glass fiber filters inserted in cylindrical apparatuses according to Ogawa (2001). NO_2 was sampled in impregnated paper filters (Whatman® n° 41) included in cylindrical apparatuses according to Machado *et al*.

(2008). Meteorological variables were continuously recorded from an automatic monitoring station (HOBO^{R} station - Mod. RX3000; Bourne, MA, USA) located next to the forests.

2.2. Metabolomic analyses

2.2.1. GC-EIMS analyzes

Leaf material (20 mg) was ground in liquid nitrogen and extracted in 500 μL of methanol/chloroform/water (12:5:1, v/v). Ribitol (50 μL) was added as internal standard (0.2 mg mL^{-1}). The extracts were placed in a dry bath for 30 min at 65°C. An aliquot (100 μL) of the upper phase (polar) was transferred to microtubes and dried under vacuum. The samples were dissolved in 28 μL of methoxyamine hydrochloride (20 mg mL^{-1}) (Sigma-Aldrich) in pyridine, vortexed and heated at 37°C for 1h. After methoxymation, the derivatives were trimethylsilylated by adding 48 μL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide, Sigma-Aldrich) and heating the mixture for 30 min at 37°C, according to a modified version of the method described by Suguiyama *et al*. (2014).

For the analysis of the nonpolar phase, an aliquot of the lower phase (100 μL) was added to 2.5 μL of tridecanoic acid (0.2 mg mL^{-1} in hexane; internal standard). The mixture was transferred to microtubes and dried under vacuum. Derivatization was performed using 25 μL of pyridine and 25 μL of BSTFA (N,O-bis-(trimethylsilyl)-trifluoroacetamide, Sigma-Aldrich), heated at 70°C for 1 h, according to a modified version of the method described by Lisec *et al*. (2006).

Samples were analyzed by Gas Chromatography coupled to Mass Spectrometry (GC-EIMS 6850/5975B Agilent Technologies) with a capillary column VF-5MS column (Agilent, length 30 m, ID 250 μm, 0.25 μm film thickness) and a pre-column (0.25 mm x 10 m). The injection volume was 1 μL using Helium as mobile phase (1.0 mL min^{-1}). Temperature was programmed as isothermal for 5 min at 70ºC, followed by a 5ºC per min ramp to 295ºC. The injector, ion source, and quadrupole temperatures were 230ºC, 200ºC, and 150ºC, respectively. The EIMS analysis employed an ionization voltage of 70 eV; the recorded mass range was of *m/z* 50 to *m/z* 600 at 2 scan s^{-1}. Substances were identified by comparing their retention times to authentic standards, and by using literature data: NIST (National Institute of Standards and Technology) digital library spectra (v2.0, 2008)

and GNPS (Global Natural Products Social Molecular Networking) spectral library (2016). The Linear Index of Retention was determined for each compound using the *n*-alkane (C_8 – C_{40}) standard according to Viegas and Bassoli (2007).

2.2.2. HPLC-DAD analyzes

Phenolic compounds were extracted from freeze-dried leaves (100 mg) using 5 mL of 80% methanol (MeOH), adjusting to a final extract concentration of 10 mg mL^{-1}. The extract was filtered (0.45 µm) and analyzed by High Performance Liquid Chromatography coupled to a diode array detector (HPLC-DAD), Agilent 1260 Analytic with Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 µm) at 45°C. The mobile phase had a constant flow of 1 mL min^{-1} and a gradient elution of 0.1% acetic acid (A) and acetonitrile (B), starting with 10% (B) for 6 min, increasing to 15% (B) for the next 1 min and maintaining for 15 min, increasing to 50% (B) for 10 min, and increasing to 100% (B) for the next 10 min, maintaining isocratic for the last 8 min (total run time 50 min). A post-run of 5 minutes was applied to return to the initial conditions according to a modified version of the method described by Santos *et al*. (2016). Phenolic compounds (flavonols and flavones) were detected at 280 and 352 nm. Contents of each compound were estimated using quercetin (1.5 to 150 µg mL^{-1}; $y=17023x - 4.5312$ and $R^2=0.9998$).

2.3. Data presentation and statistical analyzes

All data identified by GC-EIMS and detected by HPLC-DAD are presented as averages and were organized into 10 metabolite classes as presented in table 1.3: monosaccharides, disaccharides, polyols, amino acids, organic acids, fatty acids, ascorbic acid, flavonoids, terpenes, and phenolic acids.

Principal Component Analyses (PCA) using PCORD (version 6.0) were performed to summarize results and investigate what metabolite classes are relevant to distinguish metabolic responses between pioneer and non-pioneer species sampled in the four forest remnants during the rainy and dry periods.

Significant differences among metabolic responses were determined by two-way ANOVA (factor 1: forest site and factor 2: seasonal period) using Sigma Plot (version 11.0). When necessary, the data were transformed in $\log^{10}$ to reach normal distribution and equal variances. After testing the interaction of two factors, the *post-hoc* Holm-Sidak method was employed. Results were considered significant at $p < 0.05$.

Non-metric multidimensional scaling (NMDS) using PAST (version 3.0) was performed to analyze which environmental parameters could influence all the results analyzed.

3. Results

3.1. Environmental conditions

Environmental parameters varied characteristically among rainy and dry seasons in all locations. The monthly values of NO_2 concentration were higher in PEFI during rainy period (21.07 μg m^{-3}) and in MSG during the dry period (25.58 μg m^{-3}). The monthly values of O_3 concentration were higher in MSG during rainy (44.17 μg m^{-3}) and dry periods (40.49 μg m^{-3}) (Fig 1.1).

In general, the highest values of all meteorological monitored parameters were observed in the rainy period. During this period, mean values of temperature were higher in PP and MSG (25.3ºC and 24.8ºC, respectively), mean values of relative humidity were higher in PP and PEFI (97.7% and 82.5%, respectively) and higher global solar radiation was measured in MSG and PEIT (424.9 W/m^2 and 341.5 W/m^2, respectively) (Fig 1.2).

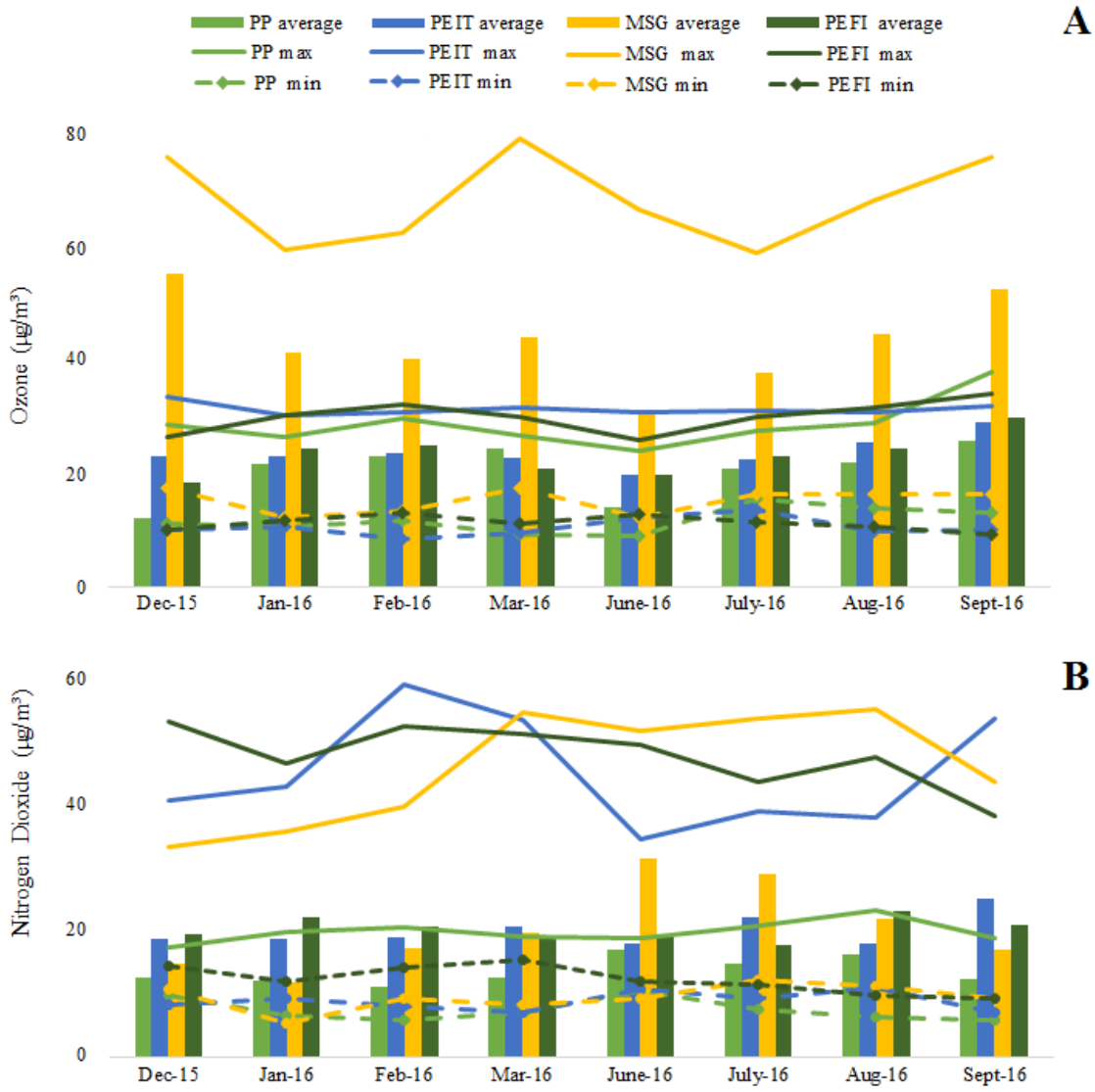

Fig. 1.1 Mean monthly concentrations of ozone (A) and nitrogen dioxide (B) in the Municipal Park Paranapiacaba (PP), State Park of Itacolomi (PEIT), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG), and State Park Fontes do Ipiranga (PEFI). Continuous and dashed lines represent the maximum (max) and minimum (min) weekly values in each month. Rainy period: December to March. Dry period: June to September.

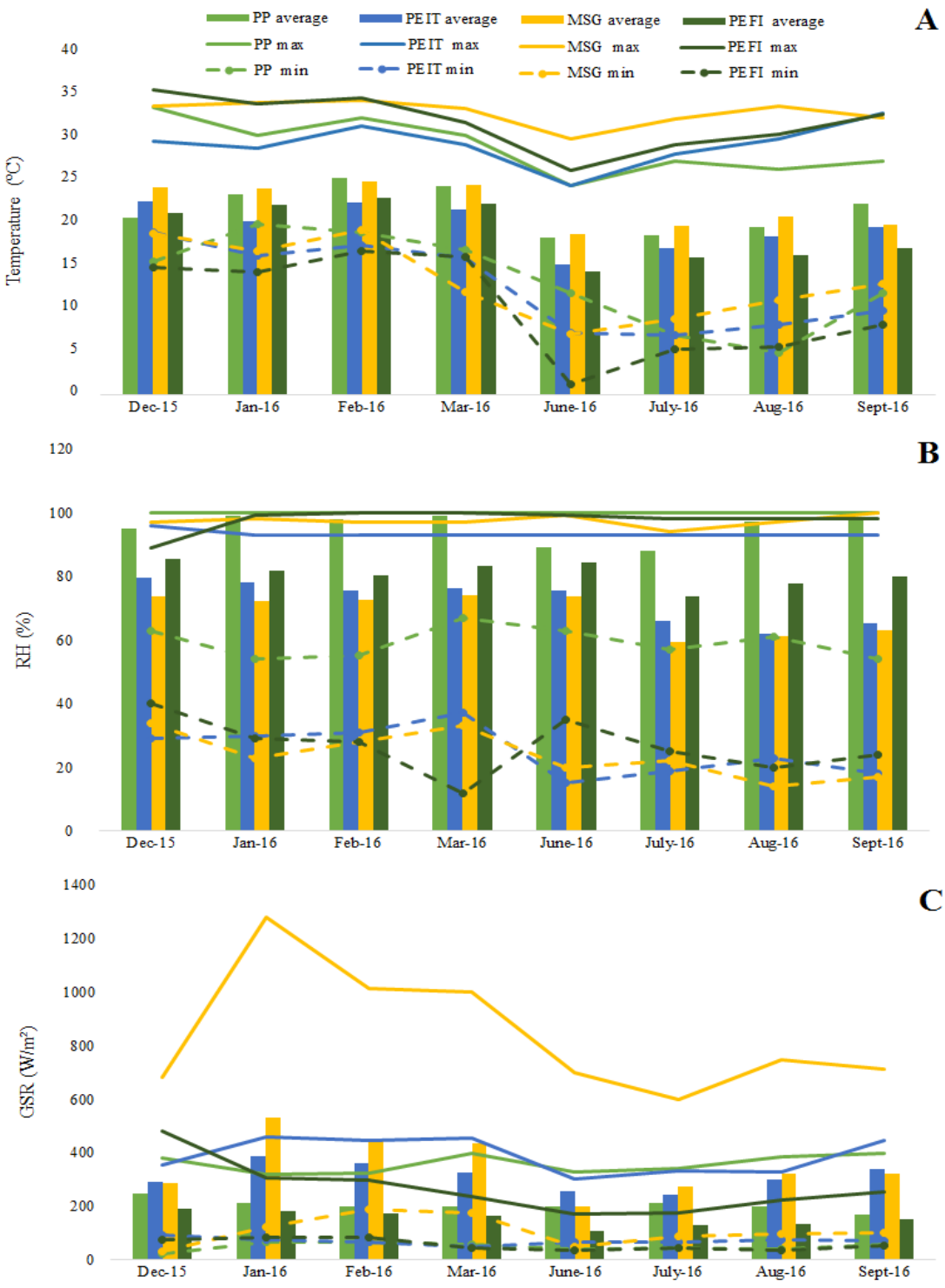

Fig. 1.2 Mean monthly values of air temperature (A), relative humidity (B), and global solar radiation (C) in the Municipal Park Paranapiacaba (PP), State Park of Itacolomi (PEIT), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG), and State Park Fontes do Ipiranga (PEFI). Continuous and dashed lines represent the maximum (max) and minimum (min) weekly values in each month. Rainy period: December to March; Dry period: June to September.

3.2. Metabolomic analysis

GC–EIMS analyses of the 21 species revealed 34 major metabolites (Tables 1.3 and 1.4), grouped into 10 classes as follow: 13 carbohydrates (5 disaccharides, 4 monosaccharides, and 4 polyols), 6 fatty acids (heptadecanoic acid, linoleic acid, palmitic acid, pentacosanoic acid, stearic acid, and octadecanoic acid), 4 organic acids (citric acid, gliceric acid, malonic acid, and oxalic acid), ascorbic acid, 3 amino acids (L-Valine, L-Threonine, and L-Serine), 3 phenolic acids (benzoic acid, caffeic acid, and shikimic acid), 2 terpenes (oleanolic acid and phytol), and 2 alkanes (dodecane and undecane).

HPLC-DAD analyses of the 21 species analyzed detected flavonoid derivatives of 2 flavone (luteolin and apigenin) and 3 flavonols (quercetin, myricetin, and kaempferol) (Table 1.4).

Principal component analyses (PCA) revealed fewer clear distinctions than expected between pioneer (P) and non-pioneer (NP) species and between the forest sites regarding the leaf contents of metabolites classes identified by GC-EIMS and HPLC-DAD (listed in Table 1.4). In contrast, the PCA identified clear distinctions between rainy and dry periods regarding the metabolomic profile of the 21 tree species (Fig. 1.3).

The PCA with data from the rainy period (Fig. 1.3A) summarized 59% of the total variability of the data in the first two axes ($p = 0.001$). Metabolite classes that showed the highest correlations with axis 1 **($r >^{+-} 0.50$)** were disaccharides, polyols, organic acids, amino acids, ascorbic acid, and terpenes. The metabolite classes that showed the highest correlation with axis 2 **($r >^{+-} 0.50$)** were monosaccharides and fatty acids. Flavonoids and phenolic acids correlated weakly with both axes (Table. 1.5).

Table 1.3. Constituents detected by GC-MS in pioneer and non-pioneer species collected in the Natural and Municipal Park Nascentes de Paranapiacaba (PP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park of Itacolomi (PEIT).

Compound	RT (min)	Cosine Index	LRI	LRI (GMD)	Suggestion
		0.93	1045	--	Tridecanoic acid (Internal
1	10.50				Standard)
2	11.04	0.87	1104	--	Oxalic acid
3	12.08	0.88	1132	--	Malonic acid
4	12.84	0.87	1489	--	Undecane
5	14.42	0.95	1211	1017.10	L-Valine
6	15.50	0.86	1248	1244	Benzoic acid
7	16.59	0.90	1288	1255	L-Serine
8	17.81	0.90	1322	1319.9	Gliceric acid
9	19.10	0.91	1759	2144.6	Heptadecanoic acid
10	20.67	0.90	2336	2243.5	Stearic acid
11	21.56	0.92	1878	2158.7	Octadecanoic acid
12	22.48	0.89	1384.01	1389	L-Threonine
13	23.22	0.92	1542	2143	Caffeic acid
14	24.91	0.92	2050	2045.4	Palmitic acid
15	26.24	0.91	1674	--	Ascorbic acid
16	26.71	0.91	1695	1700	Xylose
17	26.95	0.89	1712	1707.6	Arabitol
18	27.17	0.91	--	--	Phytol
19	27.21	0.94	1718	1721	Ribitol (Internal Standard)
20	27.71	0.88	2225	2215.6	Linoleic acid
21	29.02	0.89	1800	--	Shikimic acid
22	29.23	0.96	1810.4	1803.9	Citric acid
23	30.16	0.96	1852	1853.9	Fructose
23a	30.36	0.93	1861	1863.1	Fructose[1]
24	30.66	0.92	1875	1873.1	Galactose
25	31.74	0.93	2003	--	Glucopyranose
26	32.28	0.89	1924.2	1919.7	Sorbitol
27	33.29	0.94	2115	1880	Glucose
27a	34.83	0.89	2196	--	Glucose[1]
28	33.63	0.87	2882	--	Dodecane
29	34.31	0.92	2168	2080.2	*Myo*-Inositol
30	35.41	0.90	2211	1913.1	Mannitol
31	38.68	0.89	3882	--	Pentacosanoic acid
32	41.58	0.94	2499	2714	Sucrose
33	44.06	0.90	2661	2966.2	Galactinol
34	46.32	0.85	--	--	Oleanolic acid
35	46.66	0.95	2772	--	Melibiose
36	46.98	0.92	2678.57	--	Maltose

RT (min): retention time in minutes; Cosine Index: GNPS; Linear Retention Index; GMD: Golm Metabolome; Fructose[1] and Glucose[1]: stereoisomers.

Table 1.4. Metabolites identified by GC-EIMS and detected by HPLC-DAD and organized in classes in pioneer and non-pioneer species collected in the Natural and Municipal Park Nascentes de Paranapiacaba (PP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park of Itacolomi (PEIT).

Metabolite	Class
Ascorbic acid	**Ascorbic acid**
L-Valine	**Amino acids**
L-Threonine	
L-Serine	
Galactinol	**Disaccharides**
Maltose	
Melibiose	
Sucrose	
Xylose	
Galactose	**Monosaccharides**
Glucose	
Glucopyranose	
Fructose	
Arabitol	**Polyols**
Mannitol	
Myo–Inositol	
Sorbitol	
Heptadecanoic acid	**Fatty acids**
Linoleic acid	
Palmitic acid	
Pentacosanoic acid	
Stearic acid	
Octadecanoic acid	
Citric acid	**Organic acids**
Gliceric acid	
Malonic acid	
Oxalic acid	
Benzoic acid	**Phenolic acids**
Caffeic acid	
Shikimic acid	
Oleanolic acid	**Terpenes**
Phytol	
Apigenina	**Flavonoids**
Quercetin	
Kaempferol	
Luteolin	
Myricetin	

Samples of *Miconia cabucu* (P; from PEFI and PEIT), *Eugenia cerasiflora* (NP; from PEIT), *Machaerium villosum* (NP; from PEIT), *Eremanthus erythropappus* (P; from PEIT) and *Drimys brasiliensis* (NP; from PEIT) were grouped in the negative side of axis 1 and were more related with higher concentrations of terpenes, phenolic acids, monosaccharides and fatty acids. Samples of *Psychotria suterella* (NP; from PP), *Piptadenia gonoacantha* (P; from MSG), *Alchornea triplinerva* (P; from MSG), *Alchornea sidifolia* (P; from PEFI), and *Croton floribundus* (P; from MSG) were grouped in the positive side of axis 1 and were related with higher concentrations of flavonoids, ascorbic acid, organic acids, disaccharides, and amino acids. Samples of *Solanum granulosoleprosum* (P; from PEFI), *Astronium graveolens* (NP; from MSG), *Pleroma raddianum* (P; from PP), *Eugenia excelsa* (NP; from PEFI), *Guarea macrophylla* (NP; from PP), and *Ocotea paranapiacabensis* (NP; from PP) were located in the positive side of axis 1 and at least 90% of them were in the positive side of axis 2, being related with higher concentrations of polyols.

PCA with data from the wet period (Fig. 1.3B) summarized 52% of the total variability of the data in the first two axes ($p = 0.001$). Metabolite classes that showed the highest correlations with axis 1 **($r >^{+-} 0.50$)** were monosaccharides, organic acids, amino acids, and terpenes. The metabolites that showed the highest correlations with axis 2 **($r >^{+-} 0.50$)** were polyols, ascorbic acid and phenolic acids. Disaccharides, fatty acids and flavonoids correlated weakly with both axes (Table. 1.5).

Samples of *Drimys brasiliensis* (NP), *Eugenia cerasiflora* (NP), *Machaerium villosum* (NP), *Eremanthus erythropappus* (P) (from PEIT), *Guarea kuntiana* (NP; from MSG), *Miconia cabucu* (P; from PEFI and PEIT), *Amaioua intermedia* (NP; from PEFI), *Ocotea beulahiae* (NP; from MSG), and *Psychotria vellosiana* (NP; from PEIT) were grouped in the negative side of axis 1 and were more related with higher concentrations of terpenes and phenolic acids. Samples of *Alchornea triplinerva* (P; from MSG), *Alchornea sidifolia* (P; from PEFI), *Astronium graveolens* (NP; from MSG), *Guarea macrophylla* (NP; from PP), *Croton floribundus* (P; from MSG), and *Myrsine umbellata* (P; from PP) were grouped in the positive side of axis 1 and were related with higher concentrations of amino acids, fatty acids, flavonoids, organic acids, polyols, and ascorbic acid. Samples of *Eugenia excelsa* (NP; from PEFI), *Guarea macrophylla* (NP; from PEFI), *Piptadenia gonoacantha* (P; from MSG), *Miconia cabucu* (P; from PP), and *Pleroma raddianum* (P; from PP) were located in the positive side of axis 1 and at least

90% of them were in the positive side of axis 2 and were related with higher concentrations of monosaccharides and disaccharides.

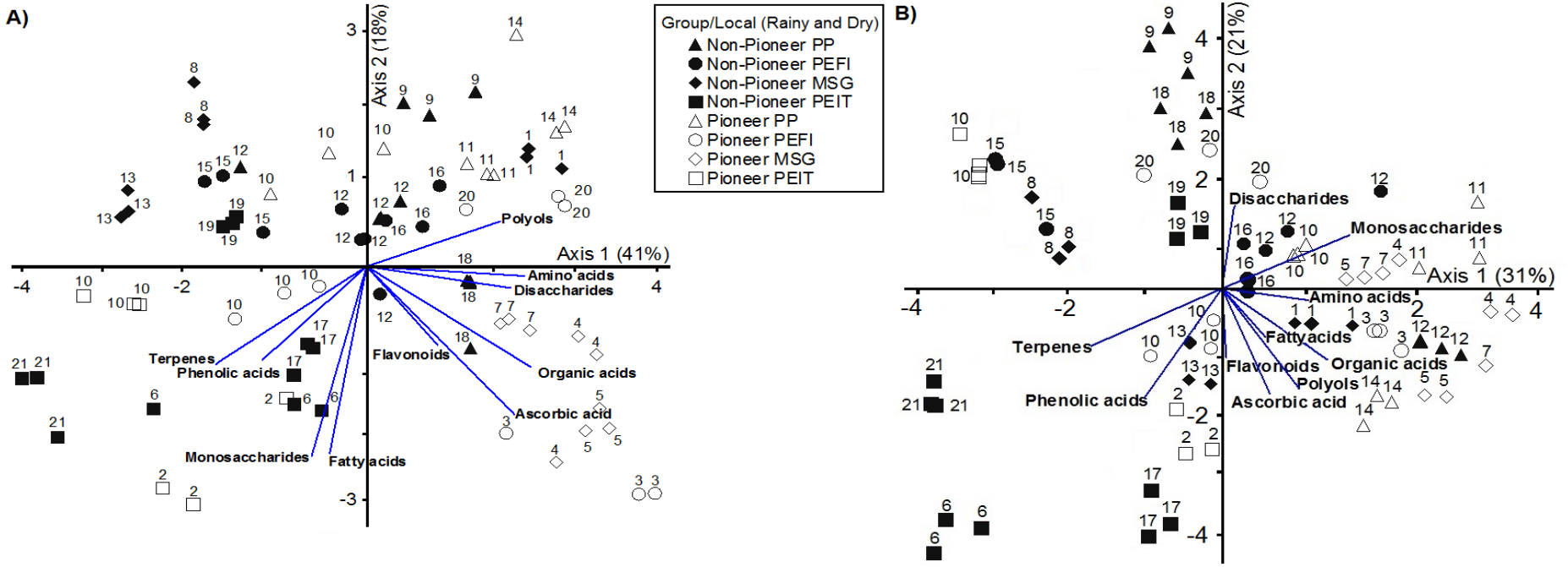

Fig. 1.3. Graphical representation of the Principal Component Analysis (PCA) of data from the rainy (A) and dry (B) periods in 2016 (n=3), including classes of compounds identified by GC-EIMS and HPLC-DAD sampled from pioneer and non-pioneer species collected in the Natural and Municipal Park Nascentes de Paranapiacaba (PP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park of Itacolomi (PEIT). (Numbers on top of the sample units correspond to the species: 1. *Astronium graveolens;* 2. *Eremanthus erythropappus*; 3. *Alchornea sidifolia*; 4. *Alchornea triplinerva;* 5. *Croton floribundus*; 6. *Machaerium villosum*; 7. *Piptadenia gonoacantha*; 8. *Ocotea beulahiae*; 9. *Ocotea paranapiacabensis*; 10. *Miconia cabucu;* 11. *Pleroma raddianum*; 12. *Guarea macrophylla*; 13. *Guarea kuntiana*; 14. *Myrsine umbellata*; 15. *Amaioua intermedia*; 16. *Eugenia excelsa*; 17. *Eugenia cerasiflora*; 18. *Psychotria suterella*; 19. *Psychotria vellosiana*; 20. *Solanum granulosoleprosum*; 21. *Drimys brasiliensis*.

Table 1.5. The table shows the correlation coefficients of each variable to axis 1 and 2. Correlation coefficients > $^{+-}$0.50 were detached.

	A) Rainy Period		B) Dry Period	
	Axis 1	Axis 2	Axis 1	Axis 2
Monosaccharides	-0.16	**-0.68**	**0.80**	0.30
Disaccharides	**0.63**	-0.08	0.07	0.45
Polyols	**0.59**	0.16	0.48	**-0.56**
Organic acids	**0.73**	-0.36	**0.66**	-0.40
Amino acids	**0.70**	-0.03	**0.54**	-0.06
Ascorbic acid	**0.65**	**-0.54**	0.30	**-0.59**
Fatty acids	-0.24	**-0.69**	0.27	-0.27
Flavonoids	0.31	-0.28	0.02	-0.39
Terpenes	**-0.68**	-0.36	**-0.82**	-0.32
Phenolic	-0.46	-0.34	-0.48	**-0.61**

Two-way ANOVA proved to have significant effects of forest sites (factor 1) on the contents of monosaccharides, disaccharides, phenolic acids, terpenes, organic acids, ascorbic acid, fatty acids, and amino acids (p=<0.001) and of seasonal periods (factor 2) on monosaccharides, disaccharides, polyols, fatty acids, ascorbic acid, flavonoids, terpenes, and phenolic acids contents (p=<0,001). The ANOVA also identified interactions between the factors in most cases, indicating that there were no significant uniform variations.

Contents of monosaccharides during the rainy and the dry seasons were significantly different among forest remnants. During the dry season, samples from PP and MSG showed the highest values of these compounds, while samples from PEIT showed the lowest contents. During the rainy season, trees from PEIT presented the highest contents of monosaccharides, while samples from PP and PEFI showed the lowest contents of these compounds. This metabolite class was more concentrated in the dry period than in the rainy period in PP, PEFI and MSG; an opposite seasonal tendency was observed in PEIT (Fig.1.4A). The content of disaccharides did not differ between the forest remnants in the rainy season and was significantly higher in the trees sampled in PP than in the other forests in the dry period (Fig.1.4B). Contents of polyols were significantly higher during the dry season than the rainy season in samples from PP, MSG, and PEIT (Fig. 1.4C).

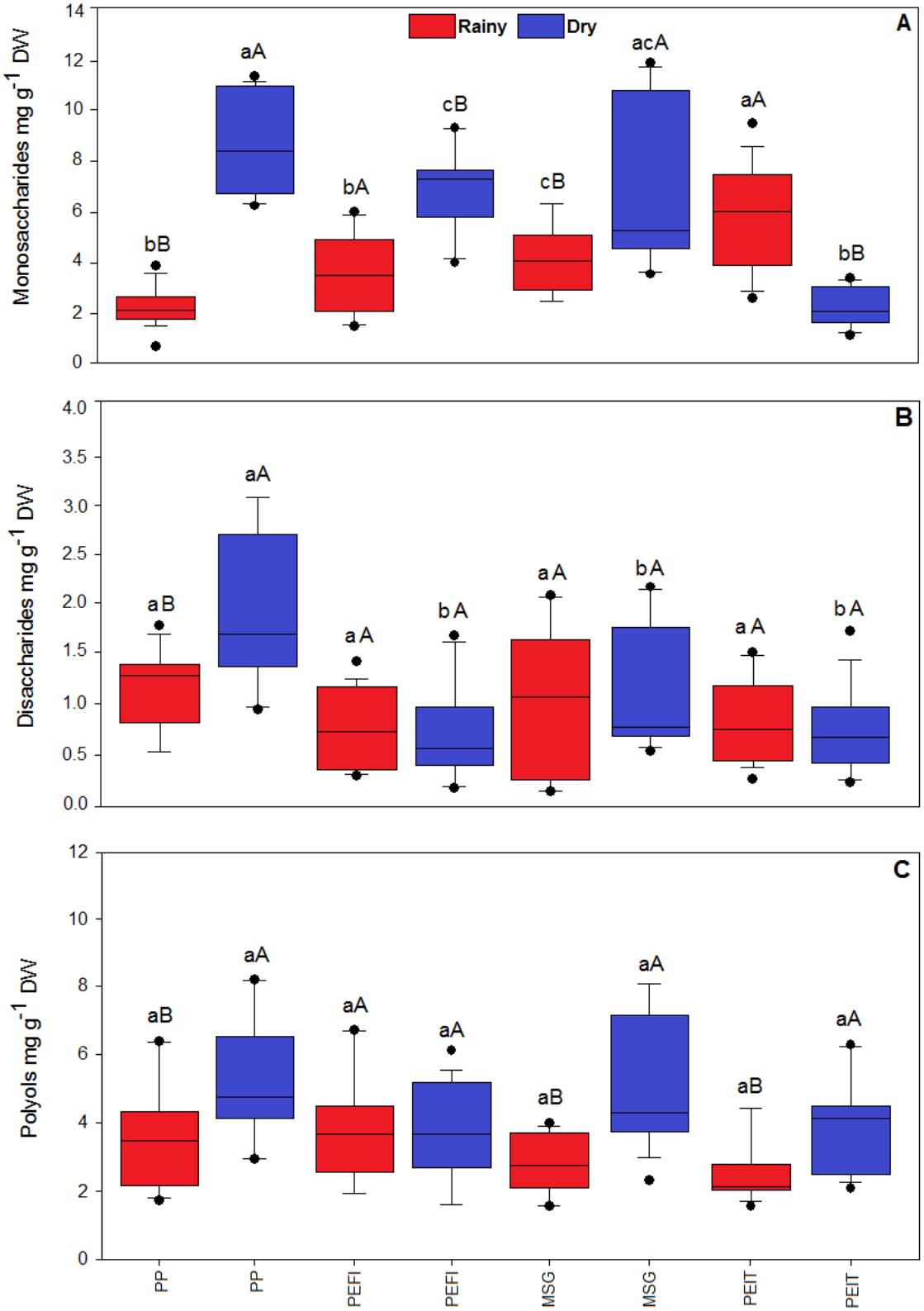

Fig. 1.4. Leaf concentrations (mg g $^{-1}$ DW) of monosaccharides, disaccharides, and polyols in species sampled in the four forest remnants during the rainy (red) and dry (blue) periods of 2016. Box plots show median, 25%- and 75%- percentile, maximum, minimum values. Lowercase letters indicate significant differences among the forest sites in each seasonal period and uppercase letters indicate significant differences among the seasonal periods in each forest site. ($p<0.05$)

Higher contents of flavonoids were measured in the trees sampled in MSG than in other forest remnants in both seasonal periods. They also differed between seasons; plants from PP, PEFI and MSG showed higher flavonoid contents in the rainy season (Fig. 1.5A). In both dry and rainy periods, the leaf levels of phenolic acids were higher in trees collected in PEIT than in samples from other sites. They were significantly higher in the dry period than in the rainy period only in plants from PEIT (Fig. 1.5C). Contents of terpenes was higher in plants sampled in PEIT than in the other forest remnants during both dry and rainy seasons. They were also higher in trees sampled during the dry period than the rainy period in PEFI, MSG, and PEIT (Fig. 1.5B).

Amino acids and organic acids were more concentrated in the trees collected in MSG than in the other forests in both seasonal periods, but no seasonal effects were observed in all forests (Figs. 1.6A and 1.6B). Contents of fatty acids did not differ in plants from all forest sites during the dry period and were higher in plants from PEIT than in samples from the other forests during the rainy period. They differed between rainy and dry seasons in trees collected in MSG and PEIT. Samples from MSG showed the highest values during dry season, while samples from PEIT showed the highest contents during rainy season (Fig. 1.6C).

The ascorbic acid concentration was more concentrated in trees collected in PEFI and MSG during the rainy season and was higher in trees collected in PP in the dry season (Fig. 1.6D).

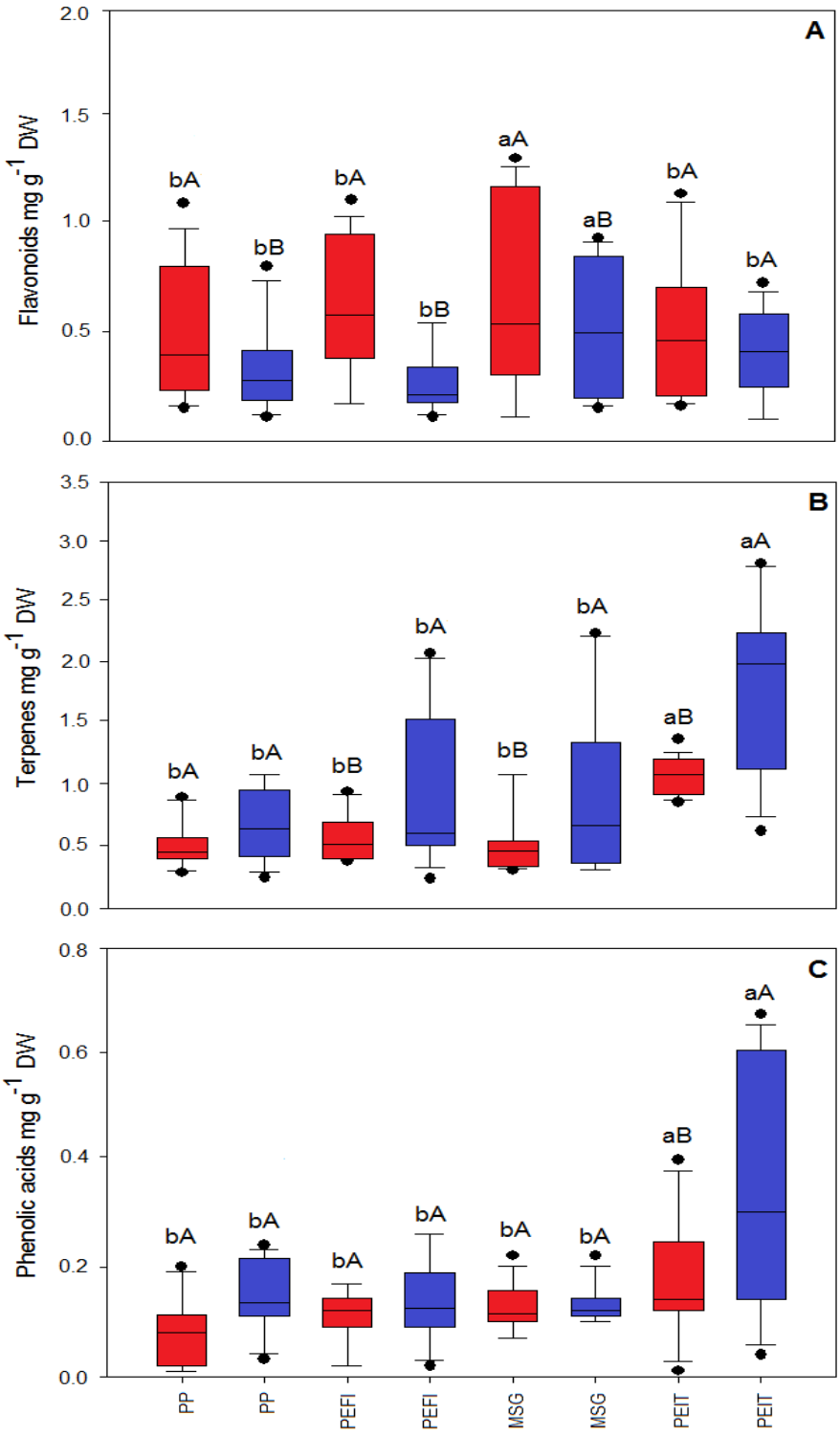

Fig. 1.5. Leaf concentrations (mg g $^{-1}$ DW) of flavonoids, terpenes, and phenolic acids in species sampled in the four forest remnants during the rainy (red) and dry (blue) periods of 2016. Box plots show median, 25%- and 75%- percentile, maximum, minimum values. Lowercase letters indicate significant differences among the forest sites in each seasonal period and uppercase letters indicate significant differences among the seasonal periods in each forest site ($p<0.05$).

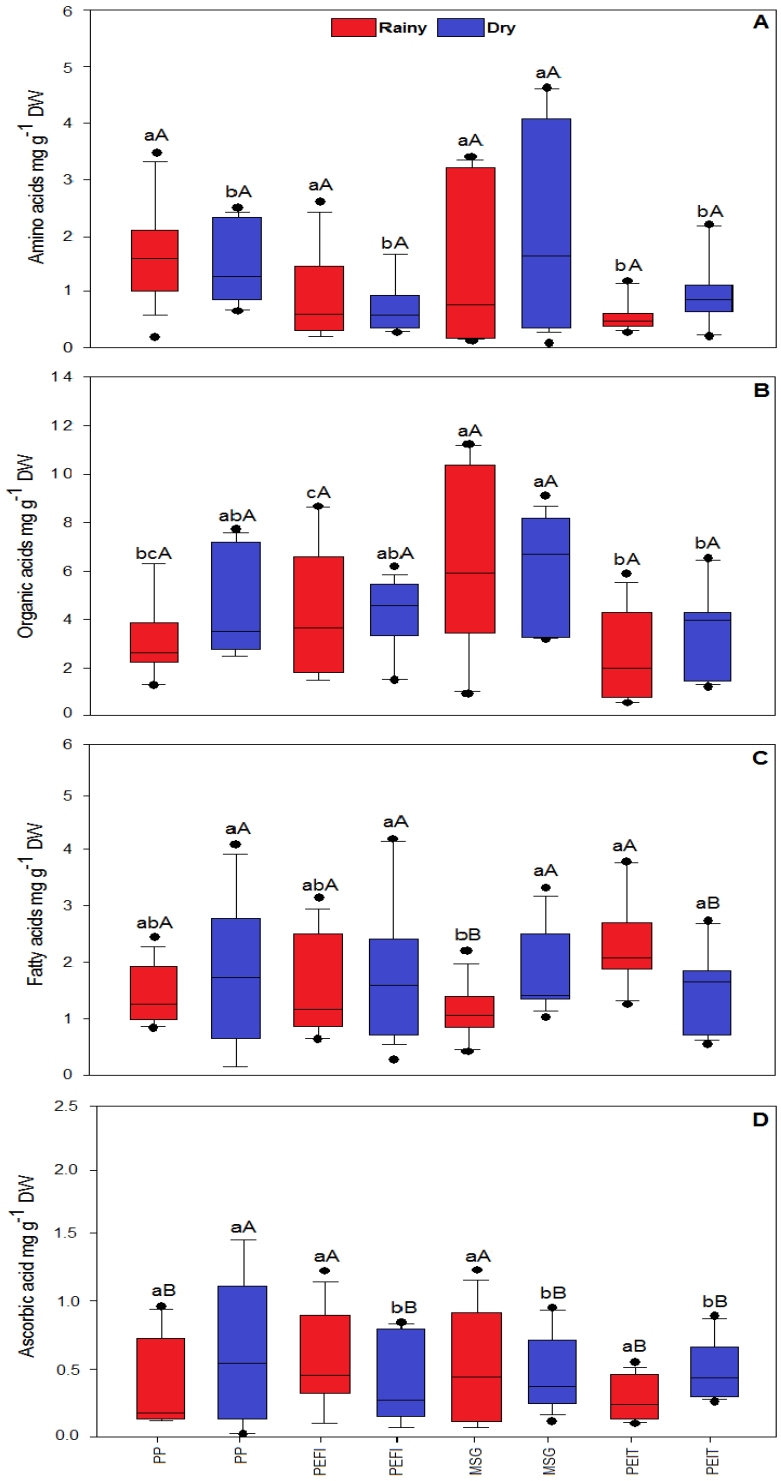

Fig. 1.6. Leaf concentrations (mg g $^{-1}$ DW) of amino acids, organic acids, fatty acids, and ascorbic acid in species sampled in the four forest remnants during the rainy (red) and dry (blue) periods of 2016. Box plots show median, 25%- and 75%- percentile, maximum, minimum values. Lowercase letters indicate significant differences among the forest sites in each seasonal period and uppercase letters indicate significant differences among the seasonal periods in each forest site ($p<0.05$).

Non-metric multidimensional scaling (NMDS) indicated the environmental parameters that influenced the leaf contents of the metabolite classes (listed in Table 1.4) identified in leaves of pioneer and non-pioneer species sampled in all forest sites. The results of NMDS analyses are displayed as scatter plots (Fig. 1.7). Analysis of similarities between the two successional groups of plants (pioneer and non-pioneer trees) indicated that different groups from the same forest remnant are in close proximity and were grouped by different environmental parameters.

During the rainy season (Fig 1.7A), the NMDS indicated that species *Miconia cabucu* (from PP and PEFI), *Psychotria suterella*, *Ocotea paranapiacabensis*, *Myrsine umbellata*, *Pleroma raddianum* (from PP), *Solanum granulosoleprosum* (from PEFI) and *Astronium graveolens* (from MSG) formed the group 1, which is more related to variations in the concentrations of NO_2. Group 2 is formed by *Eremanthus erythropappus*, *Miconia cabucu*, *Drimys brasiliensis*, *Eugenia cerasiflora*, *Machaerium villosum* (from PEIT), *Guarea kuntiana*, *Ocotea beulahiae* (from MSG) and *Amaioua intermedia*, *Guarea macrophylla* (from PEFI) and are more related to climatic variables as global radiation and relative humidity. Group 3 is formed by *Alchornea sidifolia*, *Eugenia excelsa* (from PEFI), *Alchornea triplinerva*, *Croton floribundus*, *Piptadenia gonoacantha* (from MSG), and *Guarea macrophylla* (from PP) and are more related to air temperature.

During the dry season (Fig 1.7B), the NMDS indicated that Group 1 is formed by *Ocotea paranapiacabensis*, *Miconia cabucu*, *Psychotria suterella* (from PP), *Amaioua intermedia* (from PEFI), and *Astronium graveolens* (from MSG), defined by high values of relative humidity. Group 2 is formed by *Machaerium villosum*, *Miconia cabucu*, *Drimys brasiliensis*, *Eugenia cerasiflora* (from PEIT), and *Ocotea beulahiae* (from MSG), defined by values of relative humidity. Group 3 is formed by *Croton floribundus*, *Piptadenia gonoacantha*, *Alchornea triplinerva*, *Guarea kuntiana (from MSG)*, *Myrsine umbellata*, *Pleroma raddianum*, *Guarea macrophylla* (from PP), *Alchornea sidifolia*, *Miconia cabucu*, *Solanum granulosoleprosum*, *Guarea macrophylla*, *Eugenia excelsa* (from PEFI) and *Eremanthus erythropappus*, *Psychotria vellosiana* (from PEIT) and are more related to air temperature, global radiation, O_3 and NO_2.

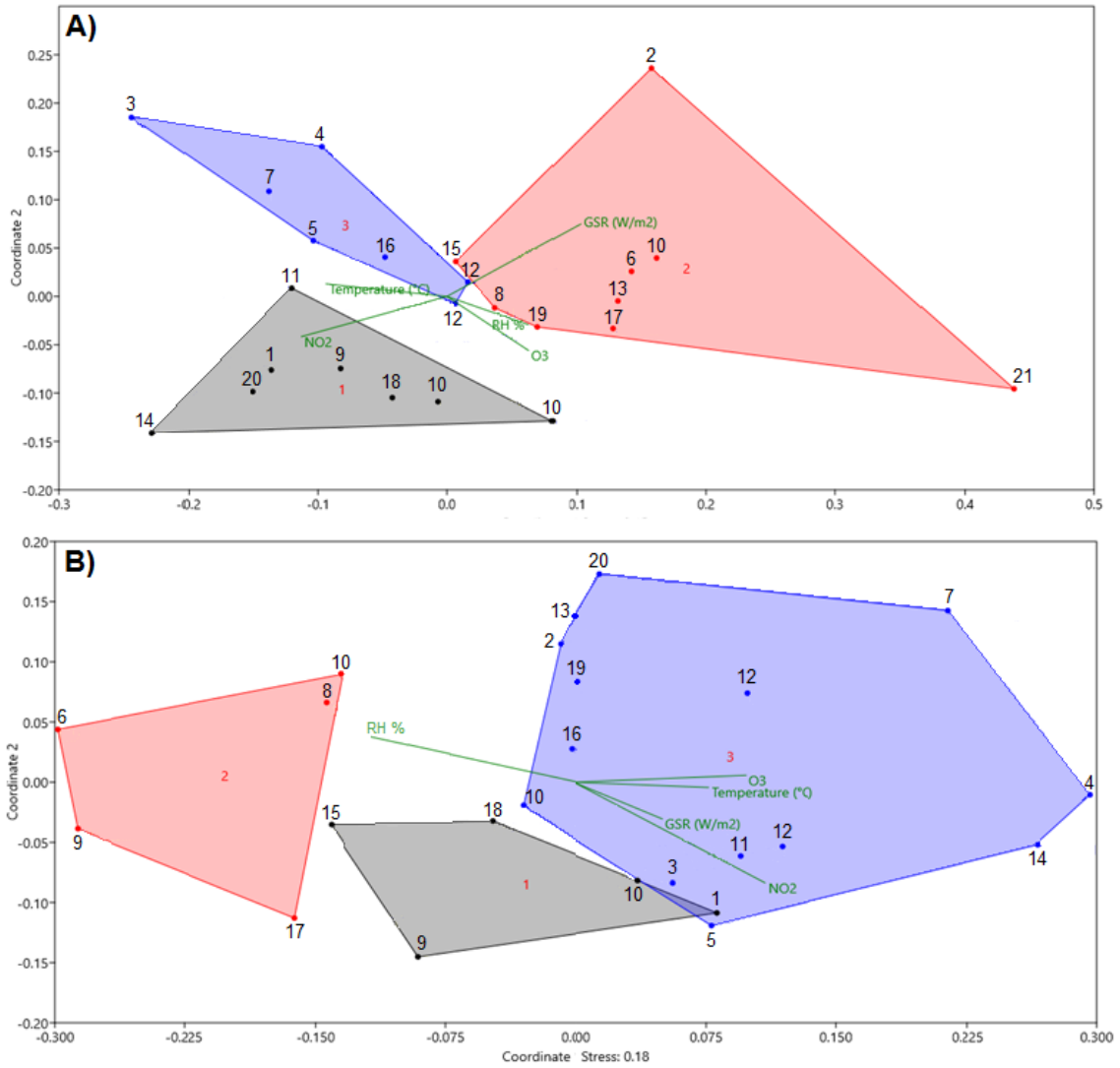

Fig. 1.7. Non-metric multidimensional scaling (NMDS) scatter plots based on pairwise distance matrix in pioneer and non-pioneer species collected in the Natural and Municipal Park Nascentes de Paranapiacaba (PP), State Park Fontes do Ipiranga (PEFI), Ecological Area of Relevant Interest Mata de Santa Genebra (MSG) and State Park of Itacolomi (PEIT) during the rainy (A) and dry (B) periods of 2016. Two coordinates are respectively pairwise distances and standard errors. Different colors of plots represent: (Grey circles): group 1; (Red circle): group 2, and (Blue circle): group 3. Numbers on top of the circles correspond to the species: 1. *Astronium graveolens;* 2. *Eremanthus erythropappus*; 3. *Alchornea sidifolia*; 4. *Alchornea triplinerva;* 5. *Croton floribundus*; 6. *Machaerium villosum*; 7. *Piptadenia gonoacantha*; 8. *Ocotea beulahiae*; 9. *Ocotea paranapiacabensis*; 10. *Miconia cabucu;* 11. *Pleroma raddianum*; 12. *Guarea macrophylla*; 13. *Guarea kuntiana*; 14. *Myrsine umbellata*; 15. *Amaioua intermedia*; 16. *Eugenia excelsa*; 17. *Eugenia cerasiflora*; 18. *Psychotria suterella*; 19. *Psychotria vellosiana*; 20. *Solanum granulosoleprosum*; 21. *Drimys brasiliensis*).

4. Discussion

The meteorological conditions during the experimental year varied among the regions included in this study, and between rainy and dry seasons. Higher values of meteorological variables were measured during the rainy season. In addition, the tree species from MSG and PEIT forests were exposed to a clearer climatic alternance between dry and rainy periods than the trees from PP and PEFI forests. In opposition to the meteorological variables, as expected, monthly mean values of NO_2 were distinguishably higher during the dry season and monthly mean values of O_3 were higher during the rainy season. The atmospheric concentrations of both air pollutants also varied between the four forest sites. Therefore, it is expected that metabolic variations observed in the tree species of these Atlantic Forest remnants occurred in response to both natural and anthropic stress factors. The non-metric multidimensional scaling analyses confirmed this assumption, mainly during the dry season. This multivariate analysis indicated that the metabolomic variations of 14 species from a total of 21 species (most of them from PEFI, MSG, and PP) were associated to combined oscillations in natural and anthropic environmental factors during this season. It is known that abiotic factors, such as temperature, global radiation, relative humidity and air pollutants can influence and change metabolic contents (Xingyuan *et al*., 2009; Liu *et al*., 2015; Večeřová *et al*., 2016; Furlan *et al*., 2020; Mpai & Sivakumar, 2020; Zhao *et al*., 2020). The question that arises is whether these alterations are metabolic markers of oxidative injury or of enhanced tolerance.

In fact, plants that grow under stress conditions such as extremes of temperature, high radiation, water availability, drought, heavy metals, soil mineral content and pollution levels, are known by their developed resistance. A high content of natural antioxidants, generally considered as resistance responses, are observed in these plants (Anwar *et al*., 2006; Gill & Tuteja, 2010; Chen & Xu, 2018). Plants activate complex molecular signals in response to environmental stresses, which may involve several organic and inorganic antioxidant molecules, including enzymes, flavonoids, carbohydrates, pigments, organic acids and ascorbic acid. They are essential in controlling plant height, root growth, increasing cuticle barriers, detecting water deficit, changing fluidity of the plasma membrane and Ca^2 influx, accumulating carbon skeletons, producing leaf surface microstructures such as trichomes and dense network of grooves, increasing density of stomata and cuticular wax layer (Hadwiger, 2009; Zhang *et al*., 2019; Ahmed *et al*., 2020; Ding *et al*., 2020; Guo *et al*., 2020; Zhang *et al*., 2020).

However, as shown by PCAs, no clear distinctions were observed between pioneer and non-pioneer species regarding their metabolomic profiles, contradicting our initial hypothesis, although they are known for distinct performances in the natural environment. It is reported in the literature that non-pioneer tree species have long longevity, limited dispersal of seeds, are adapted to shade, and have a less effective antioxidant metabolism (Batista, 2004; Favaretto *et al*., 2011; Esposito *et al*., 2018). In opposition, pioneer species are tolerant to high solar radiation and show higher photosynthetic rates, fast growth and higher biomass accumulation (Nogueira *et al*., 2004; Rappaport & Montagnini, 2014).

In summary, the PCA evidenced that the most responsive metabolite classes to environmental variations were the leaf contents of monosaccharides, polyols, flavonoids, terpenes, organic acids, ascorbic acid, and phenolic acids.

Soluble sugars are sources of carbon and energy, act as nutrients and regulators, and can also funnel NADPH producing metabolism by being a source to the oxidative pentose-phosphate (OPP) pathway that acts in ROS scavenging (Rolland et al., 2005; Keunen *et a*l., 2013; Akšić *et al*., 2015). The low contents of soluble sugar in *Drimys brasiliensis*, *Machaerium villosum*, *Miconia cabucu*, and *Eremanthus erythropappus* observed in this study could affect tissue repairing, antioxidant production and growth (Asada, 2006). In this sense, solar radiation and temperature can alter carbohydrate biosynthetic enzyme activities and increase transpiration (Gautier *et al*., 2008). Some authors reported that seasonal changes in irradiance and pollutant contaminants decrease photosynthesis and have a profound effect on plant sugar metabolism (Beckles, 2012; Engela, 2016; Nowicka *et al*., 2018; Fernandes *et al*., 2019).

A major function of the polyols involves the protection of thylakoids and the plasma membrane, and the stabilization of proteins. They also act as sinks of energy or reducing power, sources of carbon and nitrogen and scavengers of reactive oxygen species (Sairam *et al*., 2006; Duan *et al*., 2012). *Myo*-inositol is an important compound in plants; this compound and its stereo-forms have several roles, such as conjugation with molecules to form other compounds, osmotic and embryogenesis regulation (Loewus & Murthy, 2000; Luo *et al*., 2011; Valluru & Van den Ende, 2011). Inositol integrates the phospholipids, and is a messenger in different signaling pathways induced by abiotic stresses (Morales *et al*., 2019). In our study, we found that *Eugenia excelsa, Eugenia cerasiflora*, *Astronium graveolens*, *Myrsine umbellate*, and *Croton floribundus* showed higher levels of polyols, which could indicate greater resistance to different environmental stressors.

Fatty acids play different functions in plants, such as energy source, components of cell membranes, membrane fluidity, precursors of cuticular wax, antioxidant activities, and plant defense (Christensen & Kolomiets, 2011; Al Turkiman *et al*., 2015; Anselmo, 2019; Jeon *et al*., 2020; Sha *et al*., 2020). Although without statistical differences, our results demonstrate that species from PP, PEFI, and MSG tended to present more fatty acids during dry season, indicating that this class of metabolites may contribute to reducing the effects of more adverse environmental stresses during this season.

Organic acids are an important class of substances that determines the plant's efficiency in mobilizing soil nutrients (Boldt-Burisch *et al*., 2019). Also, citric acid, one of the organic acids identified in high levels in some tree species here studied, was positively correlated with increased thickness of cuticle layer, epidermis, cortex, and parenchymatous pith in pepper fruits, increasing their resistance against the *Botrytis cinerea* infection (Mekawi *et al*., 2019). In addition, some organic acids (e.g citric, malic, and oxalic acids) promote metal solubilization in soils to improve plant's phytoextraction ability (Sidhu *et al*., 2019; Zhang *et al*., 2019). Moreover, in relation to this class of substances, Sha *et al*. (2020) suggested that Ni stress redirected carbon metabolism to provide carbon skeletons for the organic acid synthesis. In this sense, Dafré-Martinelli *et al.* (2020) indicated that *Croton floribundus* and *Piptadenia gonoacantha* were characterized by high Ni concentrations, which may help to explain the high contents of organic acids in these species, as indicated by the PCAs.

In general, the stimulatory effects of organic acids during the rainy season could be attributed to the increased levels of antioxidative compounds especially enzymatic and non-enzymatic compounds to establishing a redox equilibrium in plants (Igamberdiev & Bykova, 2018; Rockett *et al*., 2020). In *Guarea macrophylla*, *Guarea kuntiana, Eugenia excelsa*, *Pleroma raddianum,* and *Alchornea triplinerva* the concentration of organic acids could be correlated to high levels of antioxidant compounds previously reported by Brandão *et al*. (2017) and Esposito *et al*. (2018) for these same species.

The increase of amino acids contents could result from protein breakdown during stress, as mentioned by Joshi *et al*. (2010). *Guarea macrophylla, Astronium graveolens, Myrsine umbellata*, *Alchornea triplinerva*, and *Piptadenia gonoacantha* showed high values of amino acids during dry season, when more stressful climatic conditions and higher NO_2 concentrations were observed. Amino acids are important protein constituents, they influence physiological processes such as plant growth and

development, intracellular pH, and act as signaling molecules (Zeier, 2013; Ros *et al*., 2014; Hildebrandt *et al*., 2015).

Ascorbic acid is a non-enzymatic antioxidant that plays a crucial role in the defense against abiotic stress acting on the detoxification of hydrogen peroxide and scavenging ROS (Fatima *et al*., 2019; Khamar & Padhy, 2019; Giampaoli *et al*., 2020; Njus *et al*., 2020). In this sense, several studies have suggested that ascorbic acid provides the first line of defense against the damage caused by air pollution (Wei *et al*., 2015, Pellegrini *et al*., 2019).

Also, plants produce secondary metabolites as a response to non-favorable environmental conditions. Flavonoids are the largest class of polyphenols that play a variety of roles in plants, such as protection against biotic and abiotic stresses (e.g. high solar irradiation), regulation of plant hormonal activity and quenching the reactive oxygen and nitrogen species (Agati *et al*., 2011; Singh *et al*., 2019; Takshask & Agrawal, 2019; Adegbola *et al*., 2020). Kumar & Pandey (2013) and Czerniewicz *et al*. (2017) suggest that many flavonoids are strong antioxidants. For example, luteolin scavenges hydrogen peroxide, whereas quercetin and apigenin probably participate in the reduction of superoxide anion radical. Our results showed that during the rainy season, when the radiation and ozone averages were high, *Eugenia excelsa, Drimys brasiliensis, Pleroma raddianum, Myrsine umbellata, Alchornea triplinerva*, and *Eremanthus erythropappus* presented 43% more flavonoids compared to the dry season, indicating that flavonoids concentration seemed to be stimulated by both high global radiation and ozone.

Dou *et al*. (2020) found a positive correlation between apigenin concentration and reactive oxygen species (ROS) scavenging rate in flowers of *Gentiana veitchiorum*. However, Verberic *et al*. (2016) verified that the content of total phenolic contents depends on the speed of the plant response, and these answers, could be different for different functional groups.

Amaioua intermedia, Astronium graveolens, Machaerium villosum, Miconia cabucu, and *Croton floribundus* showed more phenolic acids during dry season, which can be a response to more adverse environmental conditions during this season in the forest sites included in this study. Liu *et al*. (2020) demonstrated that phenolic acids, such as caffeic acid, a low molecular weight compound, have good antioxidant properties due to their phenolic hydroxyl groups and can act on the apoplast. In this sense, Brandão *et al*. (2017) reported that the accumulation level of compounds could be related to different capacities of species to compensate the oxidative stress. Several authors demonstrated

that antioxidant compounds are important in plants, they play a significant role in the defense system against reactive oxygen species (Wang *et al*., 2015; Al-Haidari & Al-Oqail, 2020). Still, phenolic acids such as benzoic acid are structural elements for other primary metabolites, including plant hormones, cofactors and defense compounds (Widhalm & Dudareva, 2015).

5. Conclusions

In summary, this study contributed to a better characterization of the metabolic profile of pioneer and non-pioneer species of Atlantic forest, allowing to infer about the function of relevant classes of metabolites in increasing tolerance against environmental stresses.

No clear distinctions were observed between pioneer and non-pioneer species regarding their metabolomic profiles. Thus, our results did not confirm our initial hypothesis. However, the metabolomic variations of 14 from 21 species studied were associated with oscillations in a combination of natural and anthropic environmental factors, especially during the dry season, demonstrating that seasonality was a strong factor for the metabolic profile.

Above all, the metabolites detected are essential for plant survival in stressful environments, are commonly found in the families of the species analyzed in this study, explaining why the species studied were not distinctly separated according to the functional group to which they belong. In relation to metabolite classes, monosaccharides, polyols, flavonoids, and organic acids seem to be the most relevant to the increased plant tolerance in the four forest remnants under different environmental stress.

Further investigations are necessary to study the different functional groups, different study sites, the seasons, and the environmental variations that the plants are subjected to.

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Chapter 2: Metabolic and physiological alterations indicate that the tropical broadleaf tree *Eugenia uniflora* L. is sensitive to ozone[2]

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Abstract

Eugenia uniflora L. is an important fruit tree native to tropical South America that adapts to different habitats, thanks to its metabolic diversity and ability to adjust the leaf antioxidant metabolism. We hypothesized that this metabolic diversity would also enable *E. uniflora* to avoid oxidative damage and tolerate the enhanced ozone (O_3) concentrations that have been registered in the (sub)tropics. We investigated whether carbohydrates, polyphenols and antioxidants are altered and markers of oxidative damage (ROS accumulation, alterations in leaf gas exchange, growth and biomass production) are detected in plants exposed to two levels of O_3 (ambient air and twice elevated ozone level in a O_3-FACE system for 75 days. Phytotoxic O_3 dose above a threshold of 0 nmol m^{-2} s^{-1} (POD0) and accumulated exposure above 40 ppb (AOT40) were 3.6 mmol m^{-2} and 14.898 ppb h at ambient, and 4.7 mmol m^{-2} and 43.881 ppb h at elevated O_3. Twenty-seven primary metabolites and 16 phenolic compounds were detected in the leaves. Contrary to the proposed hypothesis that tropical broadleaf trees are relatively O_3 tolerant, we concluded that *E. uniflora* plants are sensitive to elevated O_3 concentrations. Experimental POD0 values were lower than the critical levels for visible foliar O_3, because of low stomatal conductance. In spite of this low stomatal O_3 uptake, we found classic O_3 injury, e.g. reduction in carbohydrates concentrations; non-significant changes in the polyphenol profile; inefficient antioxidant responses; increased contents of ROS

and indicators of lipid peroxidation; reductions in stomatal conductance, net photosynthesis, root/shoot ratio and height growth. However, we also found some compensation mechanisms, e.g. increased leaf concentration of polyols for protectioning the membranes, and increased leaf number for compensating the decline of photosynthetic rate. These results help filling the knowledge gap about tropical tree responses to O_3

Keywords: Carbohydrates, flavonoids, reactive oxygen species, photosynthesis, growth and biomass, tropical species.

1. Introduction

Eugenia uniflora L. is an important broadleaf fruit tree native to tropical South America (Auricchio and Bacchi, 2003; Melo *et al*., 2007; Denardin *et al*., 2015). However, this species is widespread in several tropical and subtropical areas, from Asia to the Caribbean (Garmus *et al*., 2014) due to its high plasticity and capacity to grow in different soil and climate conditions.

Several studies indicate that *E. uniflora* polyphenol composition is effective against some bacteria, has diuretic, anti-rheumatic, antifebrile and anti-inflammatory actions and therapeutic potential for stomach diseases (Da Cunha *et al.*, 2016; Sobeh *et al.*, 2019). Furthermore, *E. uniflora* leaf extracts show high antioxidant potential related to polyphenol composition, especially flavonoids and tannin contents (Kade *et al*., 2008; Mesquita *et al*., 2017; Souza *et al*., 2018).

E. uniflora has potentially been exposed to increasing ozone (O_3) pollution throughout its occurrence area. This pollutant is the most damaging phytotoxic air pollutant because of its oxidative potential (Paoletti *et al*, 2017; Bloss, 2018). It has caused adverse effects on forest health and biodiversity all around the world, including the tropics and subtropics (Gerken *et al*., 2016; Pope *et al.*, 2019; Paralovo *et al.*, 2019; Dong *et al*., 2020; Siciliano *et al*., 2020).

The O_3 toxicity level to plants will depend on their capacity of maintaining cellular homeostasis, by mobilizing antioxidants, such as enzymes (e.g. ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase) and non-enzymatic compounds (e.g., ascorbic acid and glutathione) of the ascorbate-glutathione cycle (Aguiar-Silva *et al*., 2016; Brandão *et al*., 2017). Plants may also adjust their primary and secondary metabolisms, thus increasing their tolerance against oxidative stress (Domingos *et al*., 2015). Soluble carbohydrates (e.g., fructose, glucose and sucrose) are regulatory molecules that control gene expression related to metabolism, stress resistance, growth and development. Sugar alcohols (e.g., *myo*-inositol) exert an important role in scavenging hydroxyl radicals originated from lipid peroxidation (Peshev & Van den Ende, 2013; Du *et al*., 2018; Shu *et al*., 2019). Polyphenols, such as flavonoids (e.g. flavonols) and tannins, besides repelling herbivores and attracting pollinators (War *et al*., 2012; Tuominen, 2013; Mouradov and Spangenberg, 2014) may also scavenge free radicals (Booker *et al*., 2012; Richet *et al*., 2012; Mierziak *et al*., 2014).

Although O_3 is an important environmental issue in tropical and subtropical areas (Li *et al*., 2020; Takahashi *et al*., 2020), there is few knowledge available on the O_3 impacts in native plant species at these areas. For example, it is known that gas exchange of subtropical broadleaf species was less impaired by O_3 than that of broadleaf species from temperate climates (Li *et al*., 2017); however, the effective O_3 flux into subtropical plants has rarely been estimated (Agathokleous *et al*., 2020; Cardoso-Gustavson *et al*., 2020).

In this study, we assumed that the capacity of *E. uniflora* to occupy different habitats is derived from the ability to alter its metabolic profile. We hypothesized that such leaf traits would also enable the species to avoid oxidative damage and tolerate elevated O_3 concentrations. We investigated whether the composition and concentrations of carbohydrates and polyphenols, and levels of antioxidants are altered and whether markers of oxidative damage (ROS accumulation and alterations in leaf gas exchange, growth and biomass production) are detected in plants of *E. uniflora* growing under elevated O_3 levels in an O_3-Free-Air Controlled Exposure (O_3-FACE) system.

2. Materials and methods

2.1. Experimental design

Seedlings of *E. uniflora* (6-month-old and approx. 20 cm high) were obtained from a Brazilian nursery (23°22'18" S, 45°39'52" W) and sent to Italy in May 2017. The seedlings were then transplanted to 1.7 L pots filled with a mixture of sand: peat: nursery soil (1:1:1, v/v). Plants were irrigated every day by a drip system to avoid water stress (i.e., volumetric soil water content was maintained to the field capacity of ≈ 0.295 m^3 m^{-3}). The experiment was carried out in a last-generation 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 the exposure facility are given in Paoletti *et al*. (2017). The plants were submitted to two O_3 levels: ambient air (AA) and twice elevated ozone level (AA + O_3 x 2.0) during 75 days of summer season (from July 10th to September 25th, 2017). The system consisted of three plots per O_3 treatment; each plot was considered as a replicate (N = 3). Six pots of *E. uniflora* were maintained in each plot (totalizing 18 plants per O_3 treatment = 36 plants).

The O_3 concentration was continuously monitored by active O_3 monitors (Mod. 202, 2B Technologies, Boulder CO, USA). Global solar radiation (GSR), air temperature (Temp), relative humidity (RH) and precipitation (P) were continuously recorded by a

Watchdog station (Mod. 2000; Spectrum Technology, Inc., Aurora, IL, USA) at 2.5 m a.g.l.

2.2. Metabolite profile

Metabolite profiles were analyzed at the end of the experiment in composite leaf samples of three fully expanded and sun-exposed leaves per plant from each plot, totaling 18 composite leaf samples per O_3 treatment. The samples were stored in an ultra-freezer, at −80ºC, until analyses.

2.2.1. Compounds detected by GC-EIMS

Leaf material (20 mg) was ground in liquid nitrogen and extracted in 500 μL of methanol/chloroform/water (12:5:1, v/v). Ribitol (50 μL) was added as internal standard (0.2 mg mL^{-1}). The extracts were placed in a dry bath for 30 min at 65°C. An aliquot (100 μL) of the upper phase (polar) was transferred to microtubes and dried under vacuum. The samples were dissolved in 28 μL of methoxyamine hydrochloride (20 mg mL^{-1}) (Sigma-Aldrich) in pyridine, vortexed and heated at 37°C for 1h. After methoxymation, the derivatives were trimethylsilylated by adding 48 μL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide, Sigma-Aldrich) and heating the mixture for 30 min at 37°C, according to a modified version of the method described by Suguiyama *et al*. (2014). For the analysis of the nonpolar phase, an aliquot of the lower phase (100 μL) was added to 2.5 μL of tridecanoic acid (0.2 mg mL^{-1} in hexane; internal standard). The mixture was transferred to microtubes and dried under vacuum. Derivatization was performed using 25 μL of pyridine and 25 μL of BSTFA (N,O-bis-(trimethylsilyl)-trifluoroacetamide, Sigma-Aldrich), heated at 70°C for 1 h, according to a modified version of the method described by Lisec *et al*. (2006).

Samples were analyzed using gas chromatography coupled to mass spectrometry (GC-EIMS 6850/5975B Agilent Technologies) with a capillary column VF-5MS column (Agilent, length 30 m, ID 250 μm, 0.25 μm film thickness) and a pre-column (0.25 mm x 10 m). The injection volume was 1 μL using Helium as mobile phase (1.0 mL min^{-1}). Temperature was programmed as isothermal for 5 min at 70ºC, followed by a 5ºC per min ramp to 295ºC. The injector, ion source, and quadrupole temperatures were 230ºC, 200ºC, and 150ºC, respectively. The EIMS analysis employed an ionization voltage of 70 eV; the recorded mass range was of *m/z* 50 to *m/z* 600 at 2 scan/s. Substances were identified

and compared with authentic standards, and using NIST (National Institute of Standards and Technology) digital library spectra (v2.0, 2008) and GNPS (Global Natural Products Social Molecular Networking) spectral library (2016). The Linear Index of Retention was calculated for each compound using the alkane standard according to Viegas and Bassoli (2007).

2.2.2. Compounds detected by HPLC-DAD-MS

Phenolic compounds were extracted from the freeze-dried leaves (100 mg) using 5 mL of 80% methanol (MeOH), and the final volume of the extract was adjusted to 10 mL. The extract was filtered (0.45 μm) and analyzed by High Performance Liquid Chromatography coupled to a diode array detector (HPLC-DAD), Agilent 1260 Analytic with Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 μm) at 45°C. The mobile phase had a constant flow of 1 mL min^{-1} and a gradient elution of 0.1% acetic acid (A) and acetonitrile (B), starting with 90% (A) for 6 min, decreasing to 85% (A) for the next 1 min and maintaining for 15 min, decreasing to 50% (A) for 10 min, and decreasing to 0% (A) for the next 10 min, maintaining isocratic for the last 8 min (total run time 50 min). A post-run of 5 minutes was applied to return to the initial conditions according to a modified version of the method described by Santos *et al*. (2016). Phenolic compounds were detected at 280 and 352 nm. Contents of each compound were estimated using quercetin (1.5 to 150 μg/mL; $y=17023x - 4.5312$ and $R^2=0.9998$).

For the identification, the samples were submitted to high performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS). The equipment used was the HPLC-30AD Shimadzu coupled to the SPD-20A and Amazon Speed ETD Bruker detectors, using the Zorbax Eclipse Plus C18 (150X 4.6 nm, 3.5 mm - Agilent) column. For mass spectrometry the conditions were: ESI 500V source, 4500V capillary voltage, 27 Psi nebulizer, 325°C drying gas and 12 L min^{-1} flow. The acquisition took place in negative and positive module.

2.3. Antioxidant compounds from ascorbate-glutathione cycle

Non-enzymatic and enzymatic antioxidants were also measured on the composite samples stored at −80°C. Three analytical replicates were performed per leaf sample. Ascorbic acid was analyzed using the chromatographic method described by López *et al*. (2005) and a HPLC (Metrohm) connected to an UV–Vis detector. Glutathione content was spectrophotometrically determined according to the method described by Israr *et al*.

(2006). Ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) were analyzed by spectrophotometric UV–vis. APX and SOD activities were determined according to 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 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. Markers of oxidative damage

The following markers of oxidative damage were measured on the composite samples stored at −80°C. Three analytical replicates were performed per leaf sample and their average was used in the statistical analyses, as the statistical unit was the individual plot (n = 3 plots).

2.4.1. Indicators of lipid peroxidation (malondialdehyde and hydroperoxide conjugated diene)

The concentrations of malondialdehyde (MDA) were determined following the method proposed by Hodges *et al*. (1999) with the corrected equation proposed by Landi (2017), where the plant material was homogenized in 0.1% trichloroacetic acid containing PVPP. Trichloroacetic acid containing thiobarbituric acid was added to the supernatant, which was maintained for 30 min at 95°C in a water bath. The concentrations of hydroperoxide conjugated diene (HPCD) were obtained from leaves in ethanol (96%) by spectrophotometric UV–Vis method after a dilution of 1:15. (Levin and Pignata, 1995).

2.4.2. Reactive oxygen species

The principle of the $^{\bullet}OH$ radical assay was the quantification of the 2- deoxyribose degradation product MDA, by its condensation with thiobarbituric acid (TBA) (Lopes *et al*., 1999). The reaction mixture to determinate the H_2O_2 contents consisted of supernatant extract (leaves + trichloroacetic acid), potassium phosphate buffer (100 mM, pH 7.0) and reagent potassium iodide (KI) (Alexieva *et al*., 2001). The $O_2^{\bullet -}$ production rate was determined using the hydroxylamine oxidation method (Wang and Luo, 1990) with some modifications. Further analytical details about ROS and indicators of oxidative stress can be found in Esposito *et al*. (2018).

2.4.3. Leaf gas exchange in light-saturated conditions

Gas exchange measurements of fully expanded sun leaves (3th to 5th from the shoot tip, 1 leaf per 1 to 3 plants per replicated plot, n = 3 plots per each O_3 treatment) were carried out on 27 July and 6-7 September 2017 by a portable infrared gas analyser (CIRAS-2 PP Systems, Herts, UK) at photosynthetic photon flux density (PPFD) of 1500 µmol m^{-2} s^{-1}, ambient CO_2 concentration 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}) and stomatal conductance for water vapour (g_{sw}).

2.4.4. Growth and biomass

At the end of the exposure period, plant growth was assessed on all plants by measuring plant height, stem diameter (near the base) and total number of leaves. For measurements of height and stem diameter, a measuring tape and a digital caliper (data expressed in cm) (Digimess, São Paulo, Brazil) were used (Sá *et al*., 2014), respectively. Leaves, stems/branches and roots of each plant were then harvested, dried in an oven at 60°C and weighted in order to estimate their biomass on a dry weight basis. Shoot to root ratios were then calculated according to Moura *et al*. (2018).

2.5. Calculation of phytotoxic ozone dose (POD)

The phytotoxic ozone dose (POD), i.e. the stomatal uptake above an hourly threshold of 0 nmol m^{-2} s^{-1} along the experiment (POD0), was determined as:

$$\mathrm{POD0} = \sum_{i=1}^{n} \left(\mathrm{F_{st,i}}\right) \cdot \Delta t \qquad (1),$$

where Δt = 1 h is the averaging period, $F_{st,i}$ is the i^{th} hourly stomatal O_3 uptake (nmol m^{-2} s^{-1}), and n is the number of hours included in the calculation period. To estimate $F_{st,i}$, we applied the methodology recommended by the Mapping Manual of the Convention on Long-Range Transboundary Air Pollution for species-specific stomatal responses (CLRTAP, 2017). Stomatal conductance for O_3 (g_{sO3}) was estimated by a multiplicative stomatal conductance model (CLRTAP, 2017):

$$g_{sO3} = g_{max} \cdot f_{light} \cdot f_{O3} \cdot max\{f_{min,} \quad (f_{temp} \cdot f_{VPD})\} \qquad (2),$$

where g_{max} is the maximum stomatal conductance (mmol O_3 m^{-2} Projected Leaf Area [PLA] s^{-1}), f_{min} is the minimum stomatal conductance, f_{light}, f_{O3}, f_{temp} and f_{VPD} account for stomatal responses to photosynthetic photon flux density (PPFD), O_3 concentration, air temperature (T) and vapor pressure deficit (VPD), respectively. It is known that high O_3 concentrations may reduce g_{sO3} (Hoshika *et al*., 2020c). We therefore applied a simple linear function to explain the variation of g_{sO3} with O_3 concentration as reported in rice leaves (Oue *et al*., 2008). It is given by:

$$f_{O3} = 1 - b \cdot [O_3] \quad (3),$$

where b is a slope of the linear regression of f_{O3} and $[O_3]$ is an hourly O_3 concentration (ppb). For details of the other functions (f_{light}, f_{temp} and f_{VPD}) see CLRTAP (2017). The function describing modification of g_{sO3} by soil moisture (i.e., f_{SW}) was not used in this study because soil moisture was kept to the field capacity throughout the experiment.
Parameterization was carried out using a boundary line analysis (Alonso *et al*., 2008; Braun *et al*., 2010; Hoshika *et al*., 2012, 2020a, b). In addition to the measurements under light-saturated conditions, the daily profile (morning: 9:00 h, midday: 12:00 h, afternoon: 15:00 h) of stomatal conductance was measured on 18 July, 30 August and 8 September 2017. Pooled data (119 data points) were used to estimate the parameters. The stomatal conductance data were divided into classes with the step-wise increases for each variable as follows: 200 μmol photons m^{-2} s^{-1} for PPFD (when PPFD < 200 μmol photons m^{-2} s^{-1}, PPFD classes with 50 μmol photons m^{-2} s^{-1} steps were adopted), 20 ppb for O_3 concentration, 2°C for T and 0.2 kPa for VPD. Model functions were fitted against each variable based on 95th percentile values per each class of environmental factors. Values of g_{max} and f_{min} were calculated as the 95th percentile and 5th percentile, respectively (Hoshika *et al*., 2012; Bičárová *et al*., 2019).

2.6. Data analysis

Significant differences between treatments for all parameters were determined by Student's t-test using Sigma Plot 11.0. To assess the effects of O_3 and measuring month on leaf gas exchange, a two-way analysis of variance (ANOVA) was applied. If necessary, an appropriate transformation of the data was performed to reach normal distribution and equal variances. Results were considered significant at $p < 0.05$.

The metabolite concentrations were also log2 transformed and analyzed via the *Heatmap* tool using the *Morpheus* platform, in order to determine the ratio between the metabolites identified in leaf samples from AA and AA + O_3 x 2.0.

3. Results

3.1. Environmental conditions during the experimental period

During the experimental period, the mean daily values of air temperature, global solar radiation, relative humidity and total daily precipitation varied between 19 and 32°C, 57 and 434 W m^{-2}, 29 and 86%, and 0 and 62 mm, respectively (Fig. 2.1A). The mean daily O_3 concentrations varied between 17 - 89 ppb at ambient air and 23 - 118 ppb at elevated ozone (Fig. 2.1B). After 75 days, the accumulated exposure over a hourly threshold of 40 ppb (AOT40) reached 14898 ppb h at ambient air and 43881 ppb h at elevated O_3.

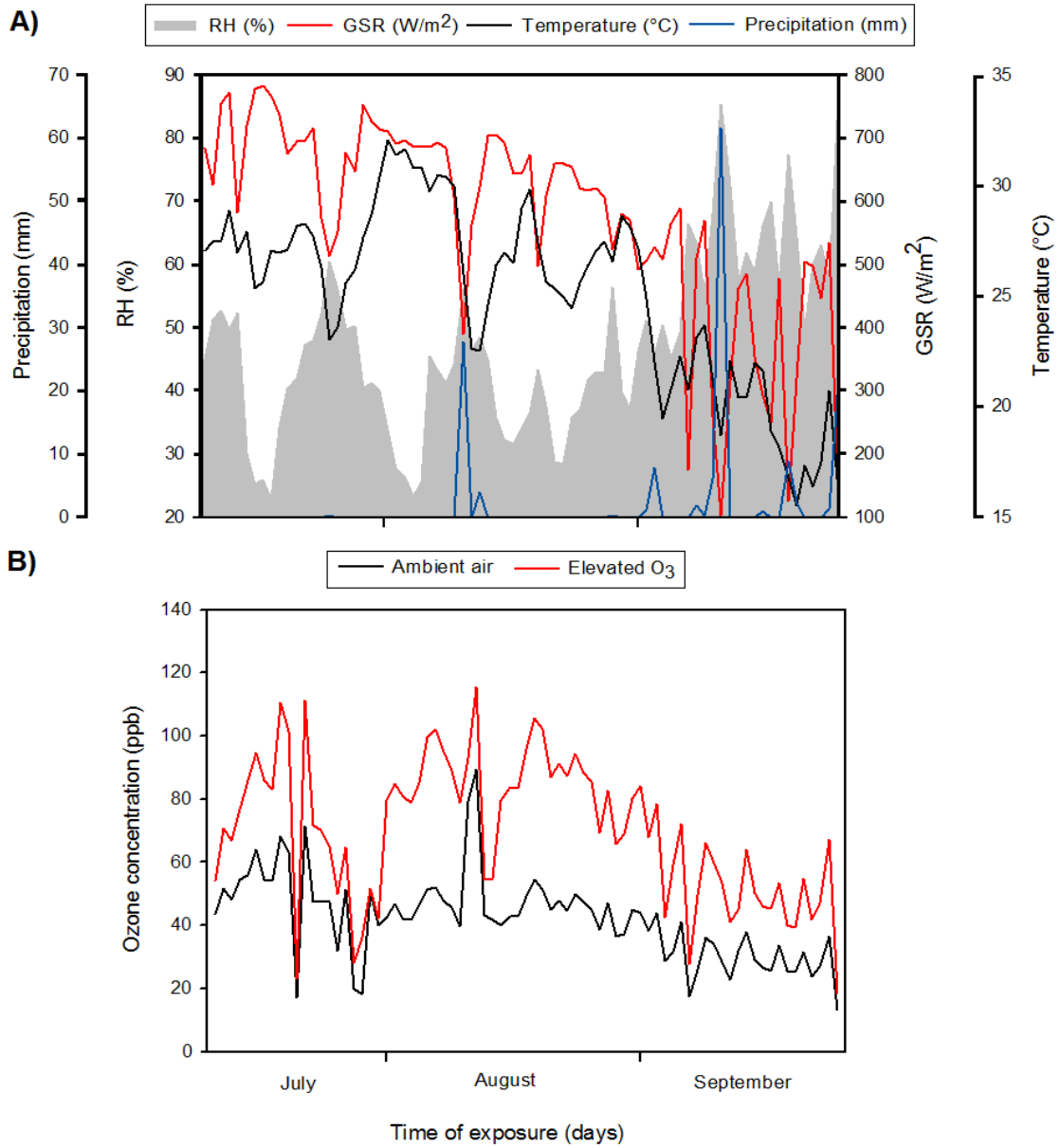

Fig. 2.1. Environmental conditions over the experimental period (from July 10th to September 25th, 2017 = 75 days of exposure). (A) Daily mean values of temperature, relative humidity (RH %), global solar radiation (GSR), total daily precipitation and (B) Ozone concentrations (ppb) at ambient air (Ozone AA) and at AA + O_3 x 2.0 (Ozone x 2.0) treatments.

3.2. Metabolite profile

3.2.1. Compounds detected by GC-EIMS

GC–EIMS analyses of *E. uniflora* leaves revealed 27 major metabolites as follow: 13 carbohydrates (6 soluble sugars and 7 sugar alcohols), 6 fatty acids, 2 organic acids, ascorbic acid, 2 amino acids, 2 phenolic compounds, and 1 alkane (Table S1 and Fig. S1;

supplementary material). Plants exposed to elevated O_3 presented significantly lower concentrations of all soluble sugars, amino acids (-26% threonine and -30% serine) and fatty acids (-38% tetradecanoic acid, -45% heptadecanoic acid, -19% octadecanoic acid and -26% stearic acid) but higher contents of all sugar alcohols than plants exposed to ambient air (Fig. 2A).

The heatmap analysis showed that the most evident differences between the treatments were observed for sucrose, glucopyranose, *myo*-inositol, ascorbic acid, heptadecanoic and tetradecanoic acid (Fig. 2B).

Table 2.1. Chromatographic data (GC-MS) of compounds from *Eugenia uniflora*.

Peak	RT (min)	Cosine Index	LRI	LRI (GMD)	Suggestion
1	10.36	0.92	1657.92	--	Tridecanoic acid (Internal Standard)
2	15.58	0.90	1258	1255	L-Serine
3	17.74	0.90	1321	1319.9	Gliceric acid
4	18.83	0.91	2153	2144.6	Heptadecanoic acid
5	20.11	0.86	2226	2215.6	Linoleic acid
6	20.67	0.90	2249.3	2243.5	Stearic acid
7	22.40	0.89	1384.01	1389	L - Threonine
8	22.65	0.91	1498	1493.3	Erythritol
9	23.26	0.92	2145	2143	Caffeic acid
10	24.01	0.91	1558.5	--	Ascorbic acid
11	24.46	0.92	2084.54	2158.7	Octadecanoic acid
12	26.93	0.88	1711	1707.6	Arabitol
13	27.17	0.94	1716.1	1721	Ribitol (Internal Standard)
14	28.42	0.91	1694	1700	Xylose
15	29.23	0.92	1810.4	1803.9	Citric acid
16	30.08	0.91	1834.4	1753.3	Tetradecanoic acid
17	30.45	0.96	1865	1853.9	Fructose
17a	30.82	0.93	1870.2	1863.1	Fructose[1]
18	31.16	0.92	1868.6	1873.1	Galactose
19	31.72	0.93	2002.2	--	Glucopyranose
20	32.66	0.89	1924.2	1919.7	Sorbitol
21	33.26	0.93	1881	1880	Glucose
22	33.64	0.87	2108.8	--	Dodecane
23	33.93	0.92	2050.2	2045.4	Palmitic acid
24	34.06	0.90	2132.1	--	Ononitol
25	34.91	0.92	2078.5	2080.2	*Myo* – Inositol
26	38.82	0.90	1922	1913.1	Mannitol
27	43.69	0.94	2494.06	2714	Sucrose
28	44.07	0.90	2961	2966.2	Galactinol
29	47.13	0.87	3036.7	3011	Epicatechin

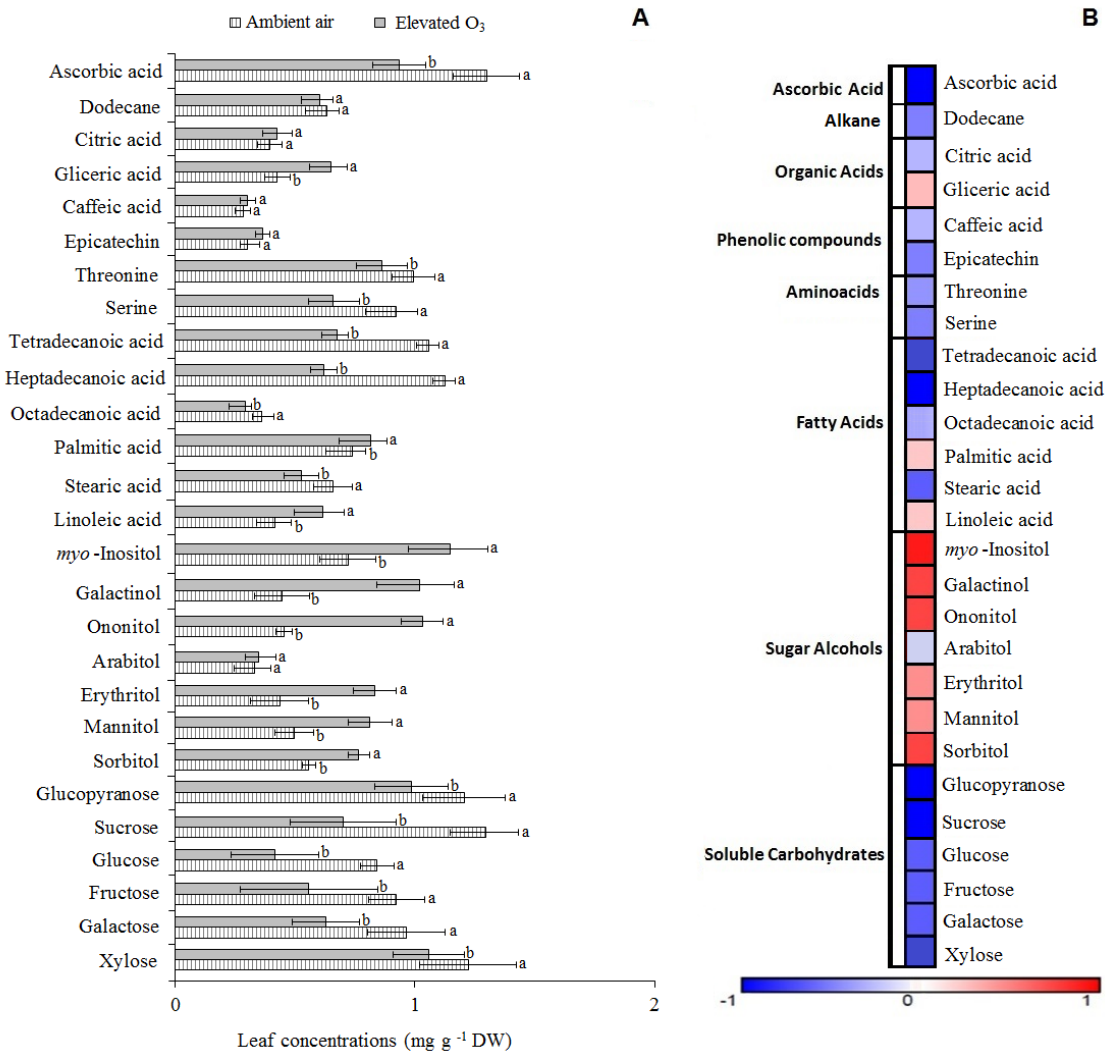

Fig. 2.2. A: Leaf concentrations of major identified compounds by GC-EIMS in *Eugenia uniflora* exposed to two treatments of ozone (AA = ambient air and AA + O_3 x 2.0 = elevated ozone). The bars represent mean ± S.E. Different letters indicate significant difference between treatments ($p < 0.05$, Student's t-test, N=3 plots). B: *Heatmap* of variations of primary metabolites. Ratios varied from ⁻1 - proportionally lower leaf concentration in plants from elevated ozone than in those from ambient air - to 1 - proportionally higher leaf concentration in elevated ozone than in ambient air and are represented by blue and red shades, respectively.

3.2.2. Compounds detected by HPLC-DAD-MS

Sixteen phenolic compounds were identified in *E. uniflora* leaves, being 14 flavonols (quercetin and myricetin derivatives), 2 cinnamic acid derivatives as caffeic acid glucoside and chlorogenic acid, and 3 non-identified compounds (Table 2.2). The foliar content of phenolic compounds did not differ between treatments (Fig. 2.3A and 2.3B).

Table 2.2. Chromatographic data (GC-MS) of compounds from *Eugenia uniflora*.

Peak	RT (min)	UV (nm)	Mass spectrum MS-MS^{-}	Mass spectrum MS-MS^{+}	Suggestion
1	1.527	224, 270	341.64 [M-H]-, 179.49 [M-162]-	--	Caffeic acid 3-glucoside
2	1.883	240, 268	--	--	N.I
3	4.236	298, 324	353.09 [M-H]-, 191.62 [M-162]-	355.12 [M+H]+	Chlorogenic acid
4	10.447	265, 286 (sh), 352	479.06 [M-H]-, 317.60 [M-162]-	481.15 [M+H]+, 319.11 [M162]+	Myricetin hexoside - 1
5	10.559	260, 298, 355	479.10 [M-H]-, 317.60 [M-162]-	481.15 [M+H]+, 319.13 [M162]+	Myricetin hexoside - 2
6	10.827	262, 294 (sh), 356	449.06 [M-H], 317.51 [M-176]-	451.12 [M+H]+, 319.09 [M132]+	Myricetin pentoside - 3
7	11.379	260, 300 (sh), 355	--	--	N.I
8	12.405	280, 300 (sh), 356	449.02 [M-H]-, 317.57 [M-132]-	451.11 [M+H]+, 319.07 [M132]+	Myricetin pentoside - 1
9	12.710	256, 304 (sh), 354	448.99 [M-H]-, 317.57 [M-132]-	451.12 [M+H]+, 319.11 [M132]+	Myricetin pentoside - 2
10	13.105	260, 270 (sh), 314 (sh), 350	463.08 [M-H]-, 317.57 [M-146]-	465.16 [M+H]+, 319.07 [M146]+	Myricetin rhamnoside
11	13.660	256, 270 (sh), 300 (sh), 354	463.08 [M-H]-, 301.29 [M-162]-	465.14 [M+H]+, 303.03 [M162]+	Quercetin glucoside - 1
12	14.414	256, 266 (sh), 352	463.08 [M-H]-, 301.29 [M-162]-	435.15 [M+H]+, 303.03 [M162]+	Quercetin glucoside - 2
13	15.993	266, 352	433.09 [M-H]-, 301.37 [M-146]-	435.14 [M+H]+, 303.10 [M146]+	Quercetin pentoside - 1
14	16.910	258, 254	433.09 [M-H]-, 301.37 [M-146]-	435.14 [M+H]+, 303.07 [M146]+	Quercetin pentoside - 2
15	20.242	256, 348	447.09 [M-H]-, 301.37 [M-146]-	449.17 [M+H]+, 303.07 [M146]+	Quercetin rhamnoside
16	26.402	266, 290 (sh), 348	615.07 [M-H]-,463.57 [M-152]-, 317.55 [M146]-	617.14 [M+H]+, 299.12 [M318]+	Myricetin rhamnosyl-gallate - 1
17	26.602	266, 348	615.28 [M-H]-,463.58 [M-152]-, 317.55 [M146]-	--	Myricetin rhamnosyl-gallate - 2
18	27.007	236, 264	--	--	N.I
19	28.335		599.17 [M-H]-, 301.30 [M-298]-	--	Quercetin rhamnosyl-gallate

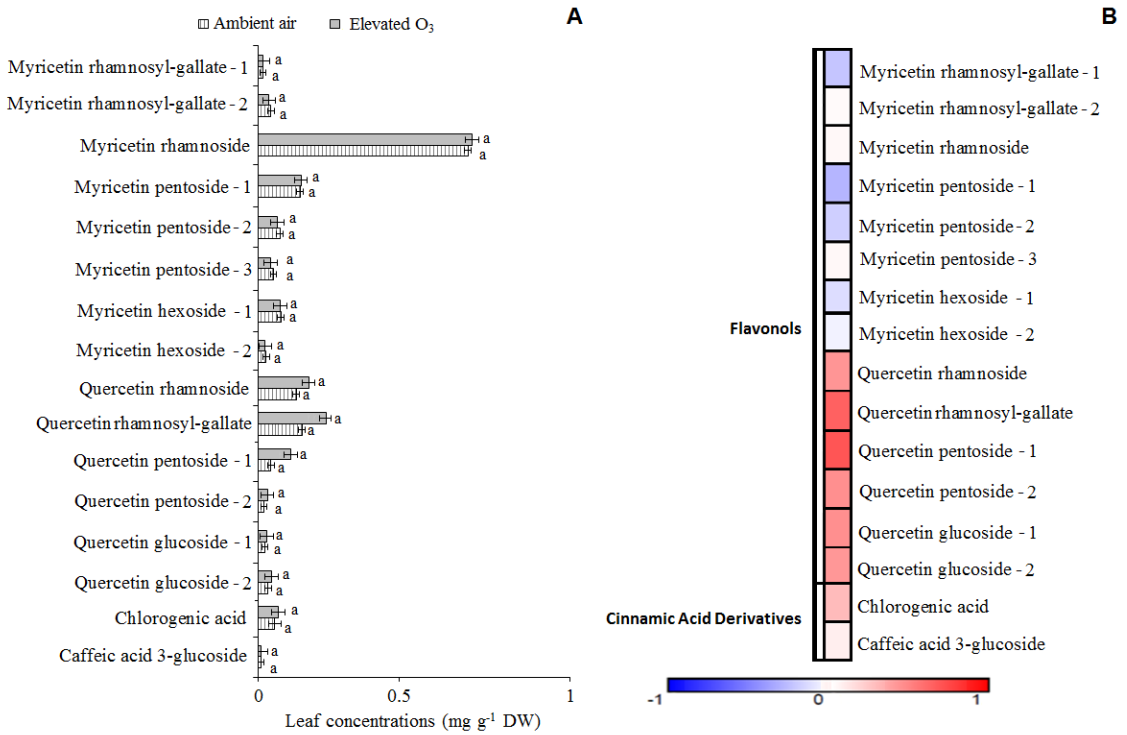

Fig. 2.3. A. Leaf concentrations of phenolic compounds identified by HPLC-MS/MS in *Eugenia uniflora* exposed to two treatments of ozone (AA = ambient air and AA + O_3 x 2.0 = elevated ozone levels). The bars represent mean ± S.E. Different letters indicate significant difference between treatments ($p < 0.05$, Student's t-test, N=3 plots). B: *Heatmap* of variations of primary metabolites. Ratios varied from -1 - proportionally lower leaf concentration in plants from elevated ozone than in those from ambient air - to 1 - proportionally higher leaf concentration in elevated ozone than in ambient air and are represented by blue and red shades, respectively.

3.2.3. Antioxidant compounds from ascorbate-glutathione cycle

The leaf content of the reduced form of non-enzymatic compounds – ascorbic acid (AsA) and glutathione (GSH) – and the activity of enzymes APX, CAT, GR, and SOD were significantly decreased in plants grown in the elevated O_3 when compared to ambient air (Fig. 2.4).

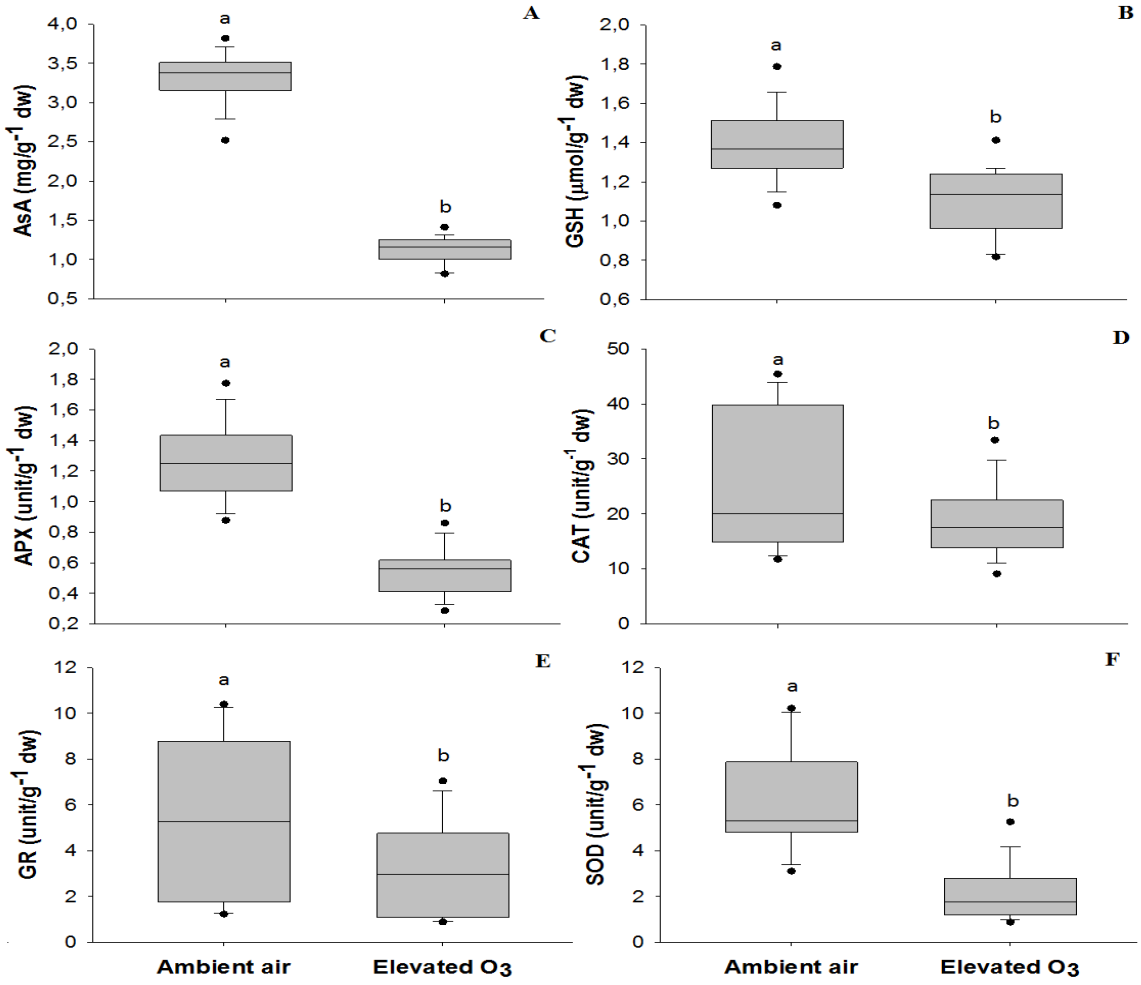

Fig. 2.4. Concentrations of non-enzymatic compounds: (**A**) ascorbic acid (AsA) and (**B**) glutathione (GSH); and activities of enzymatic compounds: (**C**) ascorbate peroxidase (APX), (**D**) catalase (CAT), (**E**) glutathione reductase (GR), and (**F**) superoxide dismutase (SOD) in leaves of *Eugenia uniflora* exposed to ambient air and elevated ozone level. Box plots show median, 25%-, 75%-percentile, maximum, minimum of values. Distinct lowercase letters indicate significant differences between treatments ($p < 0.05$, Student's t-test, n=3 plots).

3.3. Markers of oxidative damage

The contents of MDA, HPDC, OH, H_2O_2 and O_2^- increased significantly 34%, 30%, 18%, 14% and 41%, respectively, in plants exposed to elevated O_3 when compared to plants exposed to ambient air (Fig. 2.5).

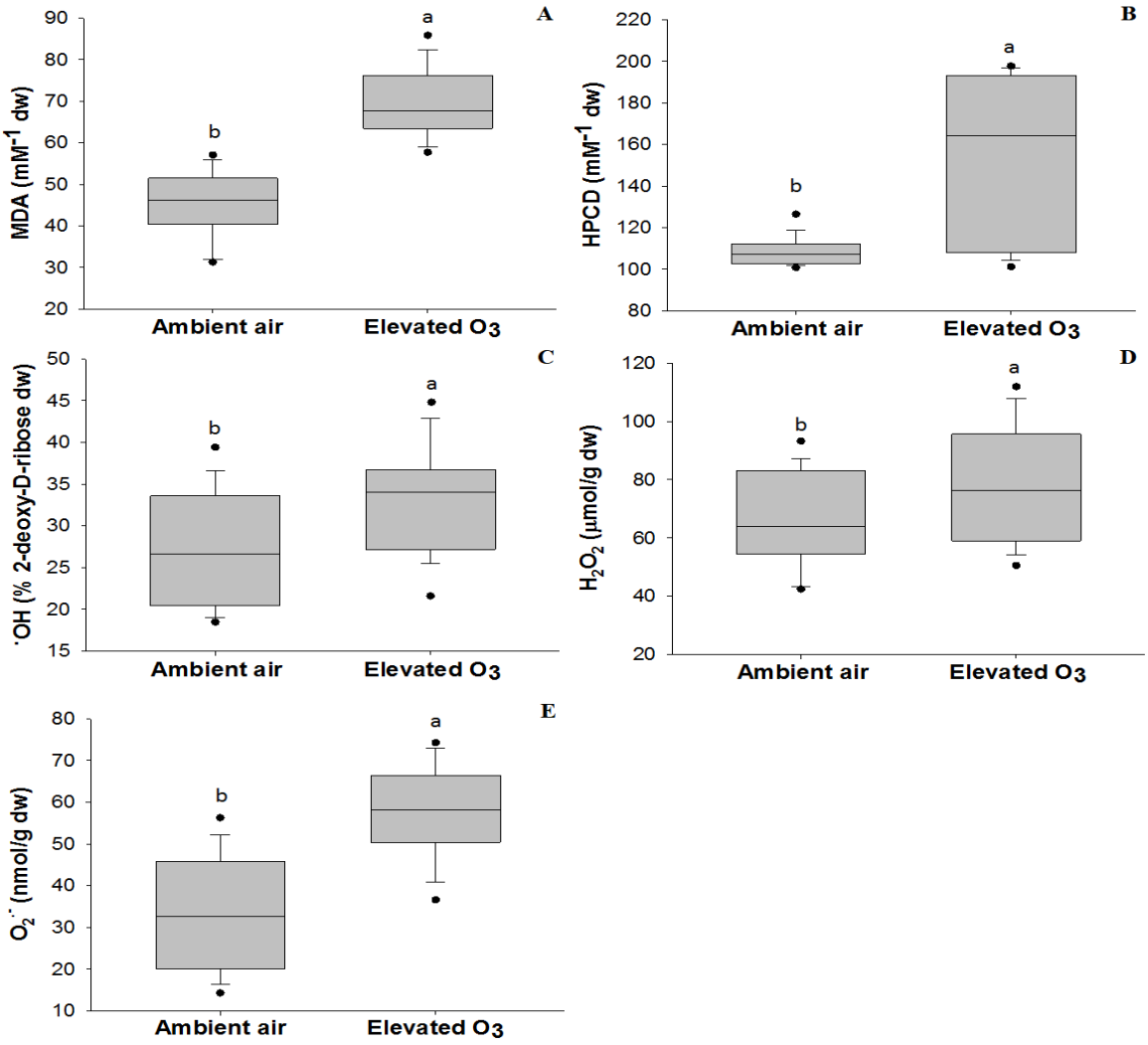

Fig. 2.5. Indicators of lipid peroxidation: (**A**) malondialdehyde (MDA) and (**B**) hydroperoxide conjugated diene (HPCD); and concentration of reactive oxygen species: (**C**) hydroxyl radical (OH), (**D**) hydrogen peroxide (H_2O_2), (**E**) and superoxide radical (O_2^-), in leaves of *Eugenia uniflora* exposed to ambient air and elevated ozone. Box plots show median, 25%-,75%-percentile, maximum, minimum of values. Distinct lowercase letters indicate significant differences between the treatments ($p < 0.05$, Student's t-test, n=3 plots).

Light-saturated net photosynthetic rate (A_{sat}) differed significantly between O_3 treatments (Fig. 2.6), with a decrease of 41% in July, and of 31% in September. In addition, *g*s decreased by 41% and 34% on average in July and September, respectively, in plants exposed to AA + O_3 x 2.0 compared to plants exposed to AA.

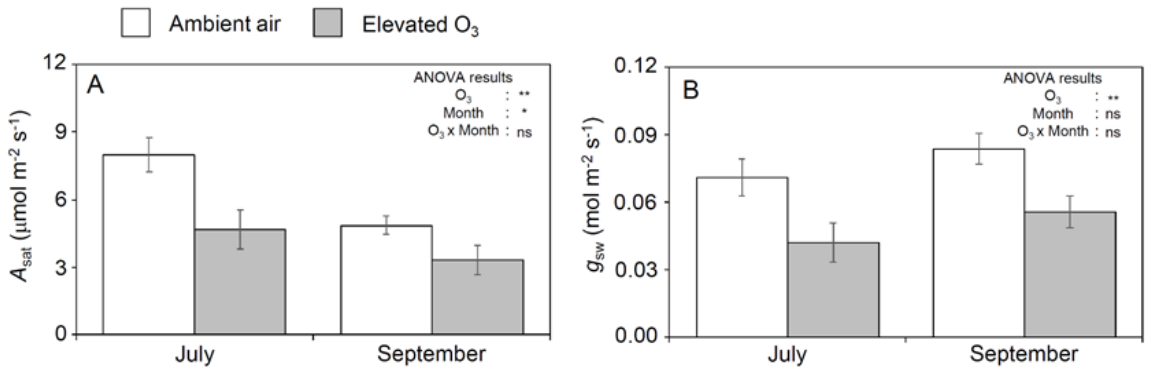

Fig. 2.6. Net photosynthetic rate (A_{sat}) and stomatal conductance (g_{sw}) of *Eugenia uniflora* in July and September 2017 under two levels of O_3 (AA, ambient O_3 concentration; AA + O_3 × 2.0, twice ambient O_3 concentration). The bars represent mean ± S.E. (n = 3 plots). Two-way ANOVA: * p<0.05, ** p<0.01, ns denotes not significant. Different letters show significant differences among treatments in each month ($p < 0.05$, Tukey test).

The leaf biomass significantly increased by 55% in plants exposed to elevated O_3 when compared to plants exposed to ambient air (Table 1). The biomass of stems and roots did not vary significantly between the O_3 treatments. However, the root to shoot ratio was decreased by 23% in the plants exposed to elevated O_3 relative to the ambient air plants. The height significantly decreased by 10% in the plants exposed to elevated O_3 when compared to the ambient air. The stem diameter did not vary significantly between the O_3 treatments. However, the number of leaves was 21% higher in the plants exposed to elevated O_3 than in the ambient air.

Table 2.3. Stomatal conductance model parameters estimated for *Eugenia uniflora*. g_{max} is maximum stomatal conductance, f_{min} is minimum stomatal conductance, *a* is a parameter determining the shape of the hyperbolic relationship of g_{sO3} response to light, *b* is a parameter to describe the variation of g_{sO3} with O_3 concentration, T_{max}, T_{opt} and T_{min} are maximum, optimal and minimum temperature for stomatal opening, VPD_{min} and VPD_{max} are the vapor pressure deficit for attaining minimum and maximum stomatal aperture (f_{VPD}).

Parameters	AA	AA + O_3 x 2.0
Biomass		
Leaves	0.67 ± 0.08 b	1.47 ± 0.06 a
Stems	2.09 ± 0.10 a	2.22 ± 0.09 a
Roots	2.57 ± 0.20 a	2.20 ± 0.20 a
Root/Shoot ratio	1.16 ± 0.07 a	0.89 ± 0.05 b
Growth		
Height	37.85 ± 0.91 a	34.20 ± 0.92 b
Stem diameter	5.53 ± 0.14 a	5.94 ± 0.19 a
Leaf number	65.16 ± 6.28 b	83.05 ± 4.30 a

3.4. Parametrization of stomatal conductance model and phytotoxic ozone dose (POD)

The parameterization of *g*s allowed to estimate a g_{max} value was of 82 mmol O_3 m^{-2} PLA s^{-1} (Table 2.4). The value of f_{min} was 12% of g_{max}. The stomatal light response followed a typical light-response curve, with a light saturation point above 1000 µmol m^{-2} s^{-1} (Fig. 2.7A), while *gs* decreased linearly with increasing O_3 concentration (Fig. 2.7B). The variation of g_s with temperature indicated an optimal temperature of 22 ˚C for stomatal opening (Fig. 2.7C). More than 1.2 kPa of VPD induced stomatal closure (Fig. 2.7D). The model estimation of g_{sO3} showed good agreement with the measurement values (Fig. 2.8) as the model was able to explain 42% of the observed g_{sO3} variance.

The POD0 values were 3.6 mmol m^{-2} at AA and 4.7 mmol m^{-2} at AA + O_3 x 2.0 during the experimental period.

Table 2.4. Stomatal conductance model parameters estimated for *Eugenia uniflora*. g_{max} is maximum stomatal conductance, f_{min} is minimum stomatal conductance, *a* is a parameter determining the shape of the hyperbolic relationship of g_{sO3} response to light, *b* is a parameter to describe the variation of g_{sO3} with O_3 concentration, T_{max}, T_{opt} and T_{min} are maximum, optimal and minimum temperature for stomatal opening, VPD_{min} and VPD_{max} are the vapor pressure deficit for attaining minimum and maximum stomatal aperture (f_{VPD}).

Stomatal conductance model parameters			*E. uniflora*
g_{max}		(mmol O_3 m^{-2} PLA s^{-1})	82
f_{min}		(fraction)	0.12
f_{light}	*a*	(constant)	0.0033
f_{O3}	*b*	(constant)	0.0039
f_{temp}	T_{opt}	($^{\circ}$C)	22
	T_{min}	($^{\circ}$C)	4
	T_{max}	($^{\circ}$C)	50
f_{VPD}	VPD_{max}	(kPa)	1.2
	VPD_{min}	(kPa)	6.6

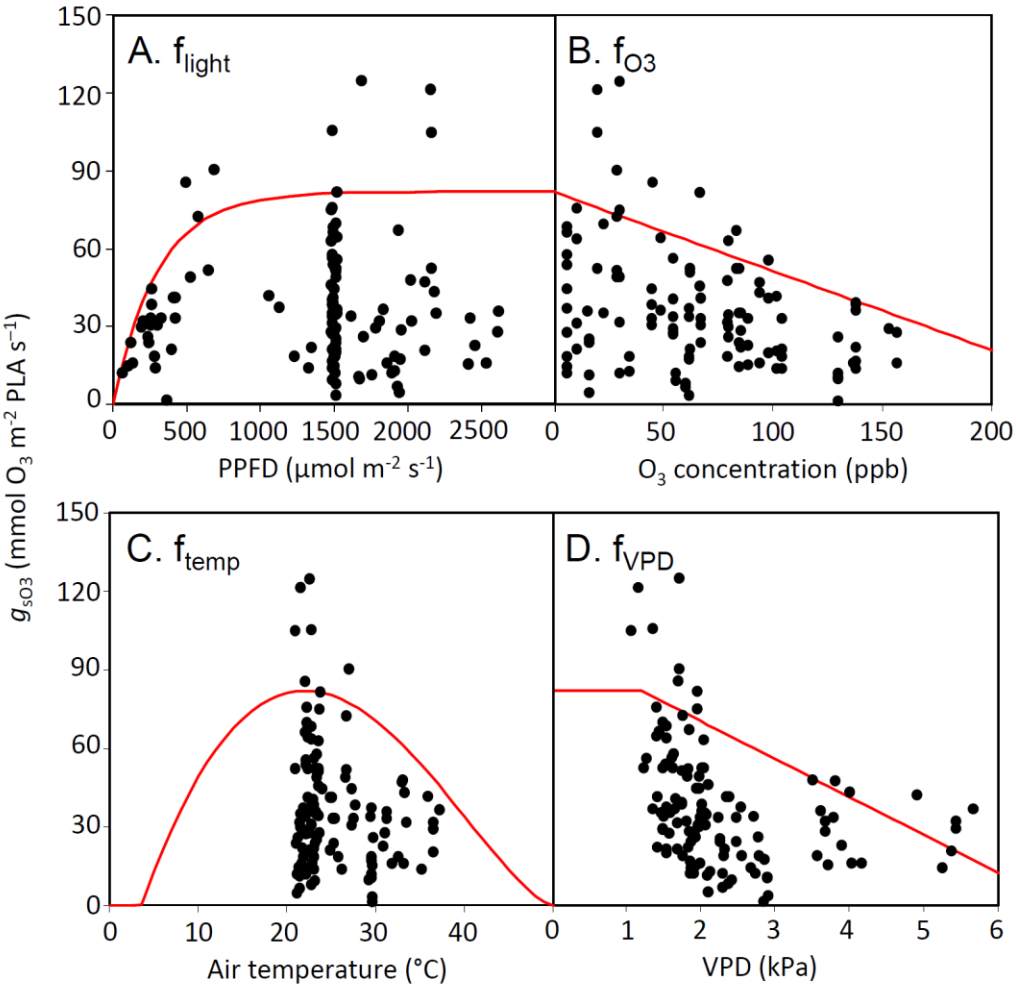

Figure 2.7. Parameterization of the stomatal conductance model for *Eugenia uniflora*. The f_{light}, f_{O3}, f_{temp} and f_{VPD} are functions of photosynthetically photon flux density (µmol photons m^{-2} s^{-1}), O_3 concentration (ppb), air temperature (T, °C) and vapor pressure deficit (VPD, kPa), respectively. The red lines are the fits to the data with the parameters listed in Table 2.

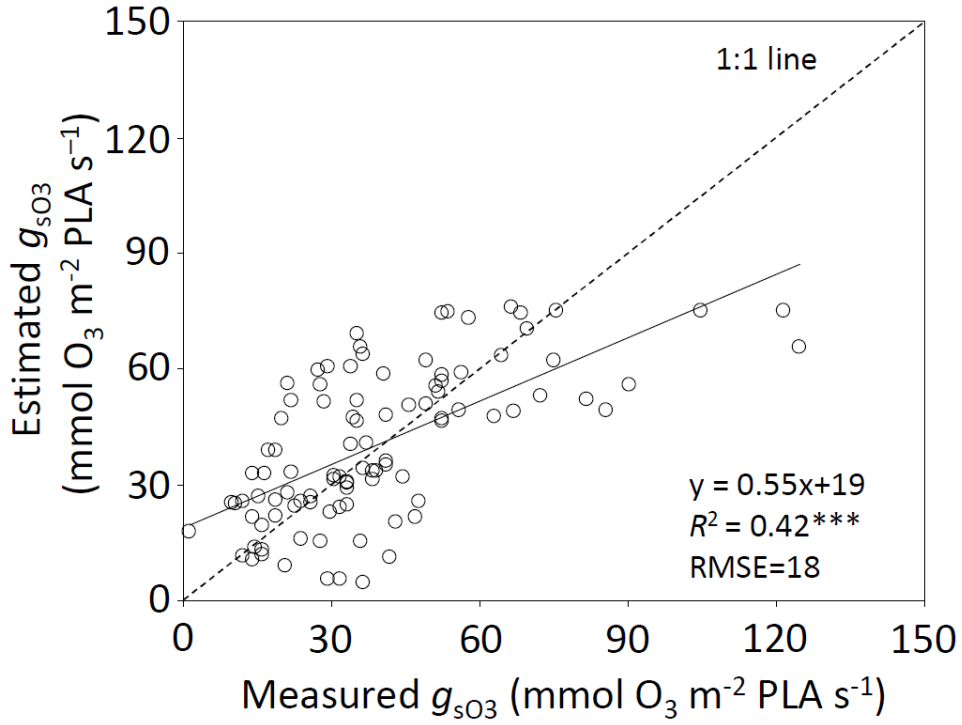

Figure 2.8 Relationship between measured and estimated stomatal conductance (g_{sO3}) in the leaves of *Eugenia uniflora*. A linear regression analysis: *** $p < 0.001$. RMSE: root mean square error (unit: mmol O_3 m^{-2} PLA s^{-1}).

4. Discussion

Exposure to elevated O_3 induced evident alterations in the concentrations of primary metabolites in leaves of *E. uniflora*. The low levels of soluble carbohydrates observed in plants exposed to elevated O_3 may compromise tissue repairing, enzymatic and non-enzymatic antioxidant production and growth (Bäck *et al*., 1999; Asada, 2006, Nishizawa *et al*., 2008), as observed in the present study.

The decrease in soluble carbohydrates seems to explain the increased leaf contents of sugar alcohols measured in *E. uniflora* exposed to elevated O_3, which may be a response of increasing resistance to oxidative stress according to Pedreschi *et al*. (2009) and Benjamin *et al*. (2018). It is known that *myo*-inositol, a polyol found in high levels in fumigated plants of *E. uniflora*, alleviates the effects of ROS (Smirnoff and Cumbes, 1989; Hu *et al*., 2018). Also, compounds derived from *myo*-inositol participate in cellular processes in plants, such as signal transduction and stress tolerance (M*et al*., 2007; Thole and Nielsen, 2008; Okada and Ye, 2009).

On the other hand, the reduction of fatty acid contents in plants growing under high O_3 concentrations is an indication of a reduced antioxidant ability of *E. uniflora*, since high contents of unsaturated fatty acids exerts an antioxidant role, favoring physiological processes of defense against free radicals (Mueller *et al*., 2006; Tsaluchidu *et al*., 2008; Yu *et al*., 2019).

Surprisingly, the polyphenol profile of *E. uniflora* did not change in response to elevated O_3, despite its known constitutive metabolic diversity (Kade *et al*., 2008; Mesquita *et al*., 2017; Souza *et al*., 2018). The major flavonoid constituent of this species was myricetin rhamnoside, which has a lower antioxidant efficiency than quercetin derivatives (Agati *et al*., 2012 Ruiz-Cruz *et al*., 2017). Therefore, the polyphenol profile, although diverse, seemed less efficient as ROS scavenger than expected.

The inefficient antioxidant responses indicated by the reduction of enzymatic and non-enzymatic compounds also revealed that *E. uniflora* was not able to compensate the oxidative stress caused by elevated O_3 levels. The lower detoxification capacity is also confirmed by the fact that photosynthetic damage per unit of O_3 uptake was rather high in this species. In fact, even though POD0 values were low and visible foliar injuries were not observed, deleterious O_3 impacts on photosynthesis were found in *E. uniflora*. In addition to

high stomatal defense capacity, we postulate that the constitutive high content of flavonoids and enhanced levels of sugar alcohols may restrict the appearance of leaf injury, as proposed by Hernández *et al*. (2009).

Increased ROS concentrations inside the cells cause membrane damage (lipid peroxidation), protein oxidation, RNA and DNA degradation, chlorophyll bleaching, and eventually lead to the destruction of the cells (Vaultier and Jolivet 2015; Choudhury *et al*., 2017; Yadav *et al*., 2019). In this study, the higher occurrence of lipid peroxidation - measured by the contents of MDA and HPCD - under elevated O_3, together with inefficient antioxidant metabolism of ascorbate-glutathione cycle, low stomatal conductance, and reduced growth in height, corroborate the assumption that *E. uniflora* is sensitive to elevated O_3 levels, contradicting the initial hypothesis. The growth and biomass allocation to different organs were altered in *E. uniflora* plants, as mentioned by several authors (Wittig *et al.*, 2009; Moura *et al*., 2018; Hoshika *et al*., 2020a). O_3 induced some alterations in biomass partitioning between above and below organs, resulting in reduced root/shoot ratio, even though 75 days of O_3 exposure may not be sufficient to accumulate significant effects on tree biomass. *E. uniflora* exposed to elevated O_3 presented an accelerated leaf turnover compared to plants exposed to ambient air, as also observed in *Alnus glutinosa* and sugarcane genotype IACSP95-5000 by Hoshika *et al*. (2020a) and Moura *et al*. (2018), respectively. The leaf turnover is an important process to compensating the decline of photosynthetic rate and support the mobilization of reserves (Hikosaka, 2005; Falster *et al*., 2018).

This species showed no leaf visible injury even after 75 days of O_3 exposure (data not shown). The flux-based approach may provide a proper assessment of O_3 injury to plants (Hoshika et al., 2020a). Sicard *et al*. (2016) and Hoshika *et al*. (2018a) suggested flux-based critical levels against leaf visible injury of 20-25 mmol m^{-2} POD0 for forest trees. However, only a few studies had been reported for modelling stomatal O_3 flux in tropical forest trees (Cassimiro *et al*., 2016). To calculate POD0, we provided for the first time the parameterization of the stomatal conductance model in *E. uniflora*. As a result, the absence of visible foliar injury in *E. uniflora* may be associated with low stomatal conductance (g_{max} = 82 mmol O_3 m^{-2} PLA s^{-1}) and O_3-induced stomatal closure (f_{O3}) which may restrict stomatal O_3 uptake, thus limiting possible O_3 damages (Hoshika et al., 2020c). In fact, *E. uniflora* leaves had lower POD0 values than the critical levels for visible foliar O_3 injury even under

elevated O_3 (POD0 = 4.7 mmol m^{-2}). Interestingly, POD0 at elevated O_3 was just 30% higher than at ambient, although twice higher mean O_3 concentrations were observed in elevated O_3.

5. Conclusions

The whole set of results led us to reject the proposed hypothesis and assume that *E. uniflora* plants are sensitive to O_3, although visible foliar injury did not occur. POD0 values were lower than the critical levels for visible foliar O_3 injury reported in the literature, as a result of low stomatal conductance observed for this species.

The hypothesis rejection was based on the following findings: reduction in soluble carbohydrates and fatty acid contents; non-significant changes in the polyphenol profile, despite its constitutive diversity, mainly regarding flavonols that are powerful antioxidants; inefficient antioxidant responses associated to the ascorbate-glutathione cycle, as indicated by the reduction of enzymatic and non-enzymatic compounds; increased contents of ROS and lipid peroxidation indicators; reduction in net photosynthesis and stomata conductance; growth reduction and reduced root to shoot ratio

Acknowledgments

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Chapter 3: How does a tropical liana species respond to nitrogen deposition and ozone? A physiological, biochemical and metabolic approach[2]

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Abstract

In recent decades, the emission of ozone (O_3) precursors in the world has significantly increased the surface O_3 levels. O_3 is considered the most phototoxic air pollutant, causing many harmful effects to vegetation, including reduction of net photosynthesis, alterations in the carbon allocation between shoots and roots, change in metabolic compounds, and reduction in growth. Nitrogen oxides and other nitrogenous compounds may be additional stress factors to natural ecosystems because they increase the amount of available N compounds in the soil through dry and wet depositions, such as nitrate (NO_3^-) and ammonium (NH_4^+). The exposure to elevated concentrations of O_3 and NO_x causes an increase in the production of reactive oxygen species (ROS), causing damage from the cellular to the ecosystem level and the intensity of these damages depends on how efficient plants are in maintaining the redox homeostasis. Equilibrium in plants may be reached by mobilizing biochemical pathways, such as the ascorbate-glutathione cycle or by adjusting their primary and secondary metabolism, including phenolic compounds and carbohydrates. *Passiflora edulis* is one of the most popular liana species and show great ability to adapt to different environments and stressors. We hypothesized that increased N deposition on soil would alleviate the O_3 effects on *P. edulis* given its high capacity to adapt to stressful environments.

[3] Capítulo a ser submetido ao periódico Ecological Indicators (QUALIS A2; IF 4.2).

The experiment was conducted for 94 days in a free-air controlled exposure (FACE), submitted to two O_3 treatments: O_3 ambient air (AAO_3), O_3 ambient air x 2.0 (AAO_3*2.0) and three potted plants per plot of each O_3 treatment received 0 kg N and other three potted plants received a solution containing 30 kg N. We investigated metabolites and signals of oxidative damage (alterations in leaf gas exchange, growth and biomass production). The whole set of results led us to accept the proposed hypothesis. In present study, the N supply alleviated the effects of elevated O_3 levels on growth, biomass, photosynthesis, chlorophyll fluorescence, while elevated the antioxidative defense components analyzed in *P. edulis*. The absence of visible foliar injury, non-reductions in growth and biomass production and significant increases in fatty acids, sugar alcohols, amino acids (such as proline), ascorbic acid, and flavonoids contents and the increase in the number of leaves, suggest that this cultivar of *Passiflora edulis* is able to tolerate the oxidative stress induced by the interactive effects of O_3 and nitrogen addition on soil.

Keywords: Carbohydrates, flavonoids, reactive oxygen species, photosynthesis, growth and biomass, liana species.

1. Introduction

Ozone (O_3) is an important secondary phytotoxic air pollutant formed in the atmosphere by complex photochemical reactions between volatile organic compounds (VOCs) and nitrogen oxides (NOx) in the presence of sunlight (Alghamdi *et al*., 2014; Bloss, 2018; Yadav *et al*., 2019; Rajabi *et al*., 2020; Touati *et al*., 2020). In recent decades, the emission of these O_3 precursors in the world, mainly by oil industries and fuel combustion, has significantly increased the surface O_3 levels (Khoramfar *et al*., 2018; Chen *et al*., 2019).

O_3 in the troposphere is considered the most phototoxic air pollutant due to its powerful oxidizing potential, causing many harmful effects to vegetation, including reduction of net photosynthesis, alterations in the carbon allocation between shoots and roots, metabolic dysfunctions, change in metabolic compounds, reduction in growth, reduction in yield quantity and quality (Ainsworth, 2017; Assis *et al*., 2018; Emberson *et al*., 2018).

Nitrogen oxides and other nitrogenous compounds may be additional stress factors to natural ecosystems because they increase the amount of available N compounds in the soil through dry and wet depositions, such as nitrate (NO_3^-) and ammonium (NH_4^+). After root absorption, these compounds are metabolized in the leaf cells by nitrate and nitrite reductase and glutamine synthase, among other enzymes of N metabolism, increasing the leaf concentrations of nitrite (NO_2^-), NO_3^-, NH_4^+, glutamine, and many other aminoacids (Buchanan *et al*., 2015; Hall, 2018; Tang *et al*., 2019). High levels of N, in both reduced and oxidized forms, may potentially cause alterations in the plant species composition and in many ecological processes (Marzuoli *et al*., 2018, Agathokleous *et al*., 2019).

Most of the polluting gases enter leaves through stomata, following the same pathway as CO_2. The exposure to elevated concentrations of O_3 and NO_x causes an increase in the production of reactive oxygen species (ROS), which will initiate multiple oxidation events, causing damage from the cellular to the ecosystem level (Moller *et al*., 2007; Esposito *et al*., 2018; Fernandes *et al*., 2019). However, the intensity of these damages depends on how efficient plants are in maintaining the redox homeostasis. Plants possess cellular antioxidants to combat oxidative stress that are able to maintain the balance and defense system of plants. This equilibrium in plants may be reached by mobilizing biochemical pathways, such as the ascorbate-glutathione cycle or by adjusting their primary and secondary metabolism, including phenolic compounds and carbohydrates (Domingos *et al*., 2015; Vaultier & Jolivet, 2015; Aguiar-Silva *et al*., 2016; Brandão *et al*., 2017; Du *et al*., 2018; Soares *et al*., 2019). Changes in the content and composition of soluble sugars, polyols, amino acids, fatty acids, carotenoids and flavonoids are generally used for assessing the potential acclimation of plant species to stress imposed by environmental factors

(Ahanger *et al*., 2018; Kapoor *et al*., 2019; Amist & Singh, 2020). Abiotic and biotic stresses, including O_3 and nitrogenous pollutants, also induce changes to plant secondary compound profiles (Caregnato *et al*., 2015; Wang & Frei, 2011), which may reduce their beneficial effects (Bortolin *et al*., 2016).

Nitrogen has been considered an important modifier of plant responses to O_3 (Zhang *et al*., 2018). It can increase O_3 uptake to levels at which antioxidant mechanisms are insufficient to counteract oxidative damage (Podda, *et al*., 2019; Shang *et al*., 2019) or can exacerbate the O_3-induced biomass loss (in an O_3-sensitive Poplar clone; Zhang *et al*., 2018). In contrast, other studies evidenced some mitigation effects due to N deposition on O_3 damages on leaves and biomass yield for different forest species (Marzuoli, *et al*., 2018). Actually, a meta-analysis performed by Feng *et al*. (2019) indicated that the current estimates of elevated O_3 impacts on plants may be under or overestimated depending on the N deposition level, plant traits and functional group. Among their conclusions, we detach: 1) the negative effects of realistic increases in O_3 concentrations (+20 to 40 ppb) on physiological traits of natural vegetation may remain unchanged by N deposition up to 60 kg ha^{-1} $year^{-1}$; 2) a N- dependent O_3 effect was significant only in perennial non- woody plants and was non- significant when only realistic increases in O_3 concentrations were considered; 3) the N addition of > 60 kg N $ha^{-1}year^{-1}$ appeared to exacerbate O_3- negative effects on photosynthesis of trees. Surely, it will also depend on the tolerance or sensitivity of plant species against excess ROS induced by both stress factors (Hayes *et al*., 2019).

In addition, most studies related by Feng *et al*. (2019) were dedicated to trees, followed by perennial or annual non-wood forbs and graminoids. The combined effects of O_3 and N deposition on lianas, such as *Passiflora* species, are still completely unknown. Lianas are a particularly important group in tropical forests and are super dominant in fragmented and disturbed forests by anthropic actions. Thereby, they have also exposed to high levels of O_3 and nitrogenous pollution in anthropized areas (Letcher & Chazdon, 2009; Pivello *et al*., 2018; Vivek & Parthasarathy, 2018; Fernandes *et al*., 2019; Birhane *et al*., 2020).

*Passiflor*a is the largest genus of Passifloraceae with about 520 species (Wohlmuth *et al*., 2010); most of them occur in the Americas (Bernacci, 2003). In controlled experiments, *Passiflora edulis* showed great ability to adapt and acclimatize to drought (Souza *et al*., 2018) and O_3 (Fernandes *et al*., 2019).

We aimed to provide new knowledge about the effects of increased levels of O_3 and N deposition on physiological, biochemical and metabolic traits of a *Passiflora* species. We raised the hypothesis that increased N deposition on soil would alleviate the O_3 effects on this species, given its high capacity to adapt to stressful environments. The experiment was conducted in a

free-air controlled exposure (FACE) facility, by exposing the plants to a combination of realistic O_3 concentrations and N deposition measured in an urban tropical area.

2. Materials and methods

2.1. Plant material

The experiment was conducted with genotypes of *Passiflora edulis* Sims f. flavicarpa O. Deg cv. IAC 273/277 donated by the Agronomic Institute of Campinas - Brazil (IAC). Seeds were germinated in petri dishes in January 2019 in the laboratory of National Research Council of Italy/Florence Research Area, following the modified procedures described in Silva (2004) and soon after the germination, the seedlings were transplanted to 17 L plastic pots containing a mixture of sand: peat: nursery soil (1:1:1) and the initial fertilization was done with 150 ml of a solution containing PK (20:20). The plants were adapted to natural conditions for one week and then positioned within the O_3 free-air controlled exposure (FACE) facility (FO_3X). Plants were irrigated every day by an individual drip system to avoid water stress.

2.2. Experimental design

The FO_3X facility is located in Florence, Italy (43°48′N, 11°12′E and 55m a.s.l.) and consists of three O_3 treatments, with three 5m×5m plots each as replicates (Fig. 3.0). The facility is described in detail by Paoletti *et al*. (2016).

The experiment lasted 14 weeks (94 days - from 17th June to 18th September 2019 - summer season). The plants were submitted to two O_3 treatments: O_3 ambient air (AAO_3) and O_3 ambient air x 2.0 (AAO_3*2.0). Three potted plants per plot of each O_3 treatment received 0 kg N ha^{-1}, i.e. 0 mg N/L of soil (N0) and other three potted plants received a solution containing 30 mg/L of soil (N30) every fifteen days, equivalent to 30 kg N ha^{-1} year^{-1}, simulating the average atmospheric N deposition in an urban area of São Paulo city, totalizing eighteen plants per O_3 and N treatment (n = 3). The N30 solution was prepared with 5 mM NH_4NO_3, according to Thomas *et al*. (1994).

Ozone concentrations at each O_3 treatment are monitored every 10 seconds by O_3 monitors (mod. 202, 2B Technologies, Boulder CO, USA). Environmental variables were continuously recorded. Global solar radiation (W/m^2), precipitation (mm), air temperature (°C), and relative humidity (RH%) were recorded by a Watchdog station (Mod. 2000; Spectrum Technology, Inc., Aurora, IL, USA).

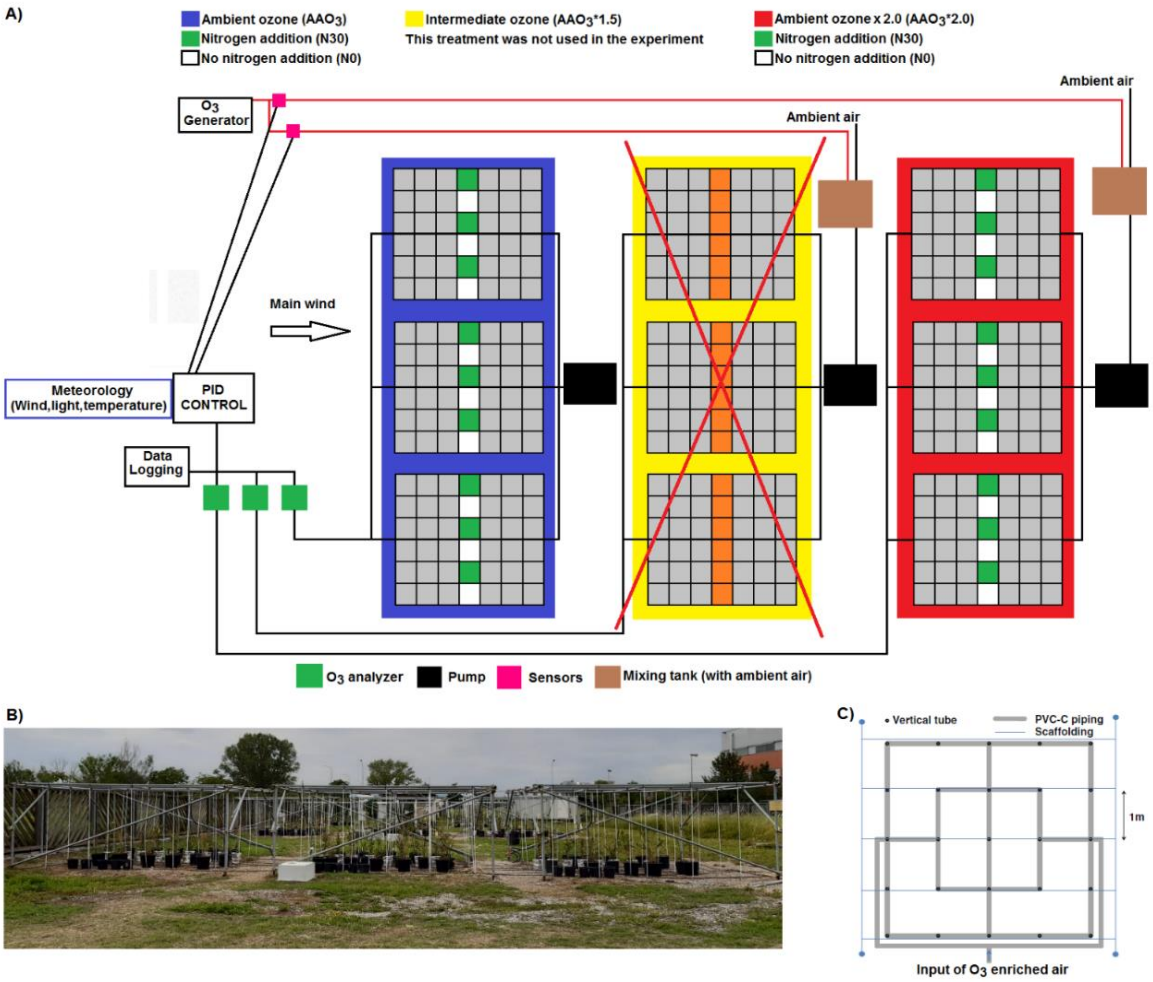

Figure 3.1. Summary description of FO_3X experimental facility located in Firenze, Italy. A) Ambient ozone (AAO3) and ambient ozone x 2.0 (AAO3*2.0) treatments, with three plots each as replicates, were included in the experiment. B) Lateral and C) above views of a plot, respectively. X: Treatment not included, adapted from Paoletti *et al*. (2016).

2.3 Physiological and biochemical responses

2.3.1 Leaf gas exchange

Gas exchange was monthly measured in fully expanded and sunny leaves (4th to 6th from the shoot tip) using a portable infrared gas analyzer (CIRAS-2 PP Systems, Herts, UK). The measurements were taken with a control value of photosynthetic photon flux density (PPFD) of 700 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 8:00 to 12:00 am (local time). 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/Ca ratio.

2.3.2 Photosystem II efficiency

At the end of the exposure period, photosystem II efficiency was measured in dark-adapted leaves by a Handy-PEA portable fluorimeter (Hansatech Instruments, Pentney, Norfolk, UK). All measurements were carried out in the morning (8:00–10:00). Three plants per treatment were selected and one of the intact leaves in the intermediate level of the plant and kept in the dark for at least 30 min in specially provided clips according to Bussotti *et al*. (2011).

2.3.3. Growth and biomass

At the end of the exposure period, plant growth was assessed by measuring plant height, stem diameter (near the base), and total number of leaves according to Sá *et al*. (2014).
For leaves, stems/branches, and roots biomass analysis each plant was harvested according to Moura *et al*. (2018).

2.3.4. Total nitrogen, nitrate and ammonium

At the end of the experiment (September 2019), leaf samples (4th to 6th order leaves from the tip of shoots) were taken for determination of total nitrogen, nitrate and ammonium contents. The leaf samples were dried in an oven at 70°C for one week and ground into fine powder. The powdered leaf samples were acid digested for eliminating organic matter. The N content were determined in the resulting extracts by micro and macroKjeldahl using a standard procedure (Horwitz, 1980) and by the colorimetric method as described by Hevia & Cioccia (1988). Concentrations of NO_3^-, NH_4^+ were determined in leaf extracts prepared with a solution of 1 mol L^{-1} KCl via microKjeldahl distillation and titration, as described in Silva *et al*. (2010).

2.3.5. Nitrate reductase and glutamine synthetase

Nitrate reductase (NR) activity was determined following Nievola & Mercier (2001), leaves (6th from the shoot tip) were collected and washed immediately with distilled water. The sample amount of 0.200 g was deposited in test tubes and submerged in 6 ml of incubation solution (previously degassed in sonicator) and was submitted under vacuum for three times of 1 min. After this procedure, the bottles were kept, in the absence of light, in a water bath at 30°C. To determine the amount of nitrite formed by the reaction, each 1 mL aliquot removed from the incubation medium was added with 0.3 mL of 1% sulfanilamide in 3 M HCl and an additional 0.3 mL of 0.02% N-naphthyl-ethylene-diamine, completing the final volume to 2.5 mL with ultrafiltered water. The readings were taken on a spectrophotometer at 540 nm. The enzyme

activity was expressed in μmol of NO_2^- released by the plant tissue in the incubation solution per hour, per gram of fresh matter.

Glutamine synthetase (GS) activity was determined according to Cammaerts & Jacobs (1985). Approximately 0.500 g of fresh leaf was extracted in Imidazole buffer solution pH 7.9 contaning 0.005 M Dithiothreitol. These samples were subjected to centrifugation at 21.000 rpm at a temperature of 4°C for 20 minutes. The total reaction volume was used enzymatic solution of 0.5 mL, consisting of: 0.1 mM imidazole buffer, whose pH was adjusted to 7.5, 49 mM hydroxylamine, 40 mM $MgCl_2$, 160 mM glutamate and 35 mM ATP. The reaction was initiated by adding 200 μL of the enzyme extract and incubating at temperature of 35°C for 1 hour. After that period the reaction was stopped with the addition of 1mL of the stop solution, consisting of: 0.123 M ferric chloride, 0.25 M HCl, and 0.1225 M trichloroacetic acid. Microtubes were prepared corresponding to time zero (T0), that is, in these the addition of the stop solution occurred after the deposition of the extract, thus interrupting the reaction. Absorbance was monitored at 540nm in a spectrophotometer. The absorbance value of T0 was discounted from the absorbance value obtained for those microtubes that remained under incubation. The enzyme activity was expressed in mmol of glutamyl gamma-hydroxamate per hour per gram of dry matter (mmol ɣGH h^{-1} g^{-1} DW).

2.3.6. Pigments

Chlorophyll (*Chl*) *a*, *b* and carotenoids (CAR) contents were determined in the same leaf extracts by spectrophotometric UV–Vis method. Sample amount of 0.150 g was ground and extracted in 10 mL of 96° (v/v) ethanol using a mortar and pestle until a colorless residue was obtained. The supernatant was analyzed 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 & De Mots, 1965).

2.3.7. Proline

Proline content was determined following Cotrozzi *et al*. (2016), with some minor modifications. The proline (Pro) was extracted from freeze-dried leaves (100 mg) using 3 mL of 80% ethanol. The homogenates were centrifuged for 15 min at 16 000 rpm at 20°C. The supernatant was filtered and 2.0 mL of the filtrate was mixed with equal volumes of glacial acetic acid (2.0 mL) and 2.0 mL of ninhydrin reagent and incubated for 1 h at 100°C. The reaction was stopped by placing the test tubes in ice. The samples were vigorously mixed with 4.0 mL toluene. After 35 seconds, the light absorption of the toluene phase was estimated at 520 nm. The proline

concentration was determined using a standard curve (2.0 to 20 μg/mL; y=0.0318x + 0.0035 and R^2=0.9976).

2.3.8. Flavonoids

Phenolic compounds were extracted from freeze-dried leaves (100 mg) using 5 mL of 80% methanol (MeOH), and the final volume of the extract was adjusted to 10 mL. The extract was filtered (0.45 μm) and analyzed by High Performance Liquid Chromatography coupled to a diode array detector (HPLC-DAD), Agilent 1260 Analytic with Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 μm - Agilent) at 45°C according to a modified version of the method described by Santos *et al*. (2016). Phenolic compounds were detected at 280 and 352 nm. Contents of each compound were estimated using quercetin (1.5 to 150 μg/mL; y=17023x – 4.5312 and R^2=0.9998).

2.4. Metabolite profile

Metabolite profile was analyzed by GC-EIMS at the end of the experiment in composite leaf samples of three fully expanded and sun-exposed leaves per plant from each plot. The composite leaf samples were stored in an ultra-freezer, at −80ºC, until analyses.

Leaf material (20 mg) was extracted in 500 μL of methanol/chloroform/water (12:5:1, v/v) and 50 μL of ribitol (0.2 mg mL^{-1}) added as internal standard, according to a modified version of the method described by Suguiyama *et al*. (2014). Samples were analyzed using gas chromatography coupled to mass spectrometry (GC-EIMS 6850/5975B Agilent Technologies) after being derivatized using MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide, Sigma-Aldrich). Substances were identified and compared with authentic standards, and using NIST (National Institute of Standards and Technology) digital library spectra (v2.0, 2008) and GNPS (Global Natural Products Social Molecular Networking) spectral library (2016). The Linear Index of Retention was calculated for each compound using the alkane standard according to Viegas & Bassoli (2007).

2.5. Statistical analyzes

Significant differences in physiological and biochemical responses between O_3 and N treatments were determined by two-way ANOVA (factor 1: ozone treatment and factor 2: nitrogen treatment) using Sigma Plot (version 11.0). Significant differences in metabolites were determined by one-way ANOVA. When necessary, the data were transformed in $\log^{10}$ to reach normal distribution and equal variances. After testing the interaction of two factors, the *post-hoc* Holm-Sidak method was employed. Results were considered significant at $p < 0.05$.

3. Results

3.1. Environmental conditions

During the experimental period, the mean daily values of air temperature, global solar radiation, relative humidity and total daily precipitation varied between 20 and 40°C, 34 and 354 W m^{-2}, 30 and 89%, and 0 and 20 mm, respectively (Fig. 3.2A).

The mean daily O_3 concentrations varied between 12 - 74 ppb at AAO_3 and 21 - 130 ppb at AAO_3*2.0 (Fig. 3.2 B). After 94 days, the accumulated exposure over an hourly threshold of 40 ppb (AOT40) reached 20473 ppb h at AAO_3 and 65389 ppb h at AAO_3*2.0.

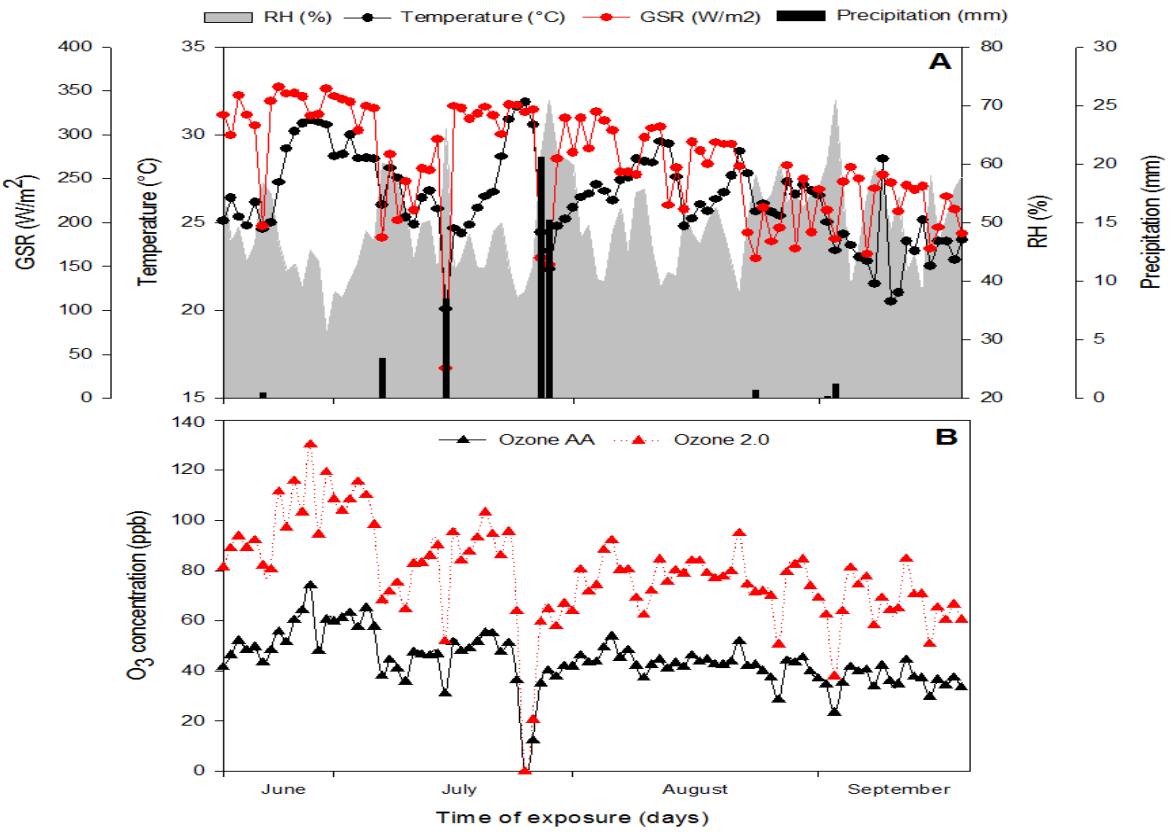

Fig. 3.2. Environmental conditions over the experimental period (from June 17th to September 18th, 2019 = 94 days of exposure). (A) Daily mean values of temperature (°C), relative humidity (RH %), global solar radiation (GSR) and total daily precipitation (mm); (B) Ozone concentrations (ppb) at ambient air (AAO_3) and at O_3 x 2.0 (AAO_3*2.0) treatments.

3.2 Physiological and biochemical responses

Two-way ANOVA identified significant effects of O_3 on: general and monthly average A_{sat}; monthly g_{sw}; general average Ci/Ca ratio; photosystem II efficiency; stem biomass and leaf' diameter and number; total nitrogen, nitrate, and ammonium contents; nitrate reductase and glutamine synthetase activities; carotenoid, proline, and flavonoid contents.

Two-way ANOVA also identified significant effects of N addition on soil on: general and monthly average A_{sat}; monthly g_{sw}; general average Ci/Ca ratio; leaf number; total nitrogen, nitrate, and ammonium contents; nitrate reductase and glutamine synthetase activities; flavonoid contents.

One-way ANOVA identified significant effects of O_3 and N addition on soil in metabolite contents.

Interactions between O_3 and N addition on soil were only proved for average A_{sat} measured in September and g_{sw} measured in August and September.

The main effects on the physiological and biochemical responses of *P. edulis* plants to O_3 and N fertilization will be described below.

3.2.1. Leaf gas exchange

Average values of A_{sat} and Ci/Ca ratio for the whole period were significantly reduced in the plants exposed to AAO_3*2.0 compared to average values obtained in plants exposed to AAO_3, regardless the N level applied on soil. In contrast, the average A_{sat} and Ci/Ca were significantly higher in plants supplied with N in excess (N30) than in plants not supplied with N (N0) in both O_3 treatments. The average values of g_{sw} for the whole experimental period were not statistically different in plants exposed to both levels of O_3 and N fertilization (Table 3.1).

Monthly average values of A_{sat} were significantly reduced in the plants exposed to AAO_3*2.0 compared to average values obtained in plants exposed to AAO_3 during the whole experimental period, regardless the N level applied on soil. The increased A_{sat} in response to N supply was evident in July and August under ambient O_3 (AAO_3). Under elevated O_3 (AAO_3*2.0), the N supply reduced the average A_{sat} measured in plants at the beginning (July) and increased at the end (September) of the experiment (Fig. 3.3A). The highest monthly values of g_{sw} were measured in plants not supplied with N and exposed to ambient O_3 (AAO_3+N0) and the lowest monthly values were found for the plants exposed in AAO_3*2.0+N30. In general, the most evident effects of O_3 and N supply on g_{sw} were evidenced in July (Fig. 3.3B). The lowest values of *A*sat and *g*s were in September (Fig. 3.3A and B).

Table 3.1. Average values (n = 3) of A_{sat} (light-saturated net photosynthetic rate), g_{sw} (stomatal conductance for water vapour) and Ci/Ca (ratio of intercellular to ambient CO_2 concentrations) in *Passiflora edulis* plants exposed to two ozone levels and two N doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30) for 94 days. Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	A_{sat} (μmol m^{-2} s^{-1})	g_{sw} (mol m^{-2} s^{-1})	Ci/Ca ratio
AAO_3+N0	6.0 Ba	147 Aa	0.42 Ba
AAO_3+N30	6.5 Aa	154 Aa	0.47 Aa
AAO_3*2.0+N0	4.8 Bb	145 Aa	0.37 Bb
AAO_3*2.0+N30	6.3 Ab	149 Aa	0.44 Ab
	TWO WAY ANOVA (*p* values)		
O_3	**0.026**	0.309	**0.002**
N	**0.003**	0.324	**<0.001**
O_3 x N	0.107	0.881	0.746

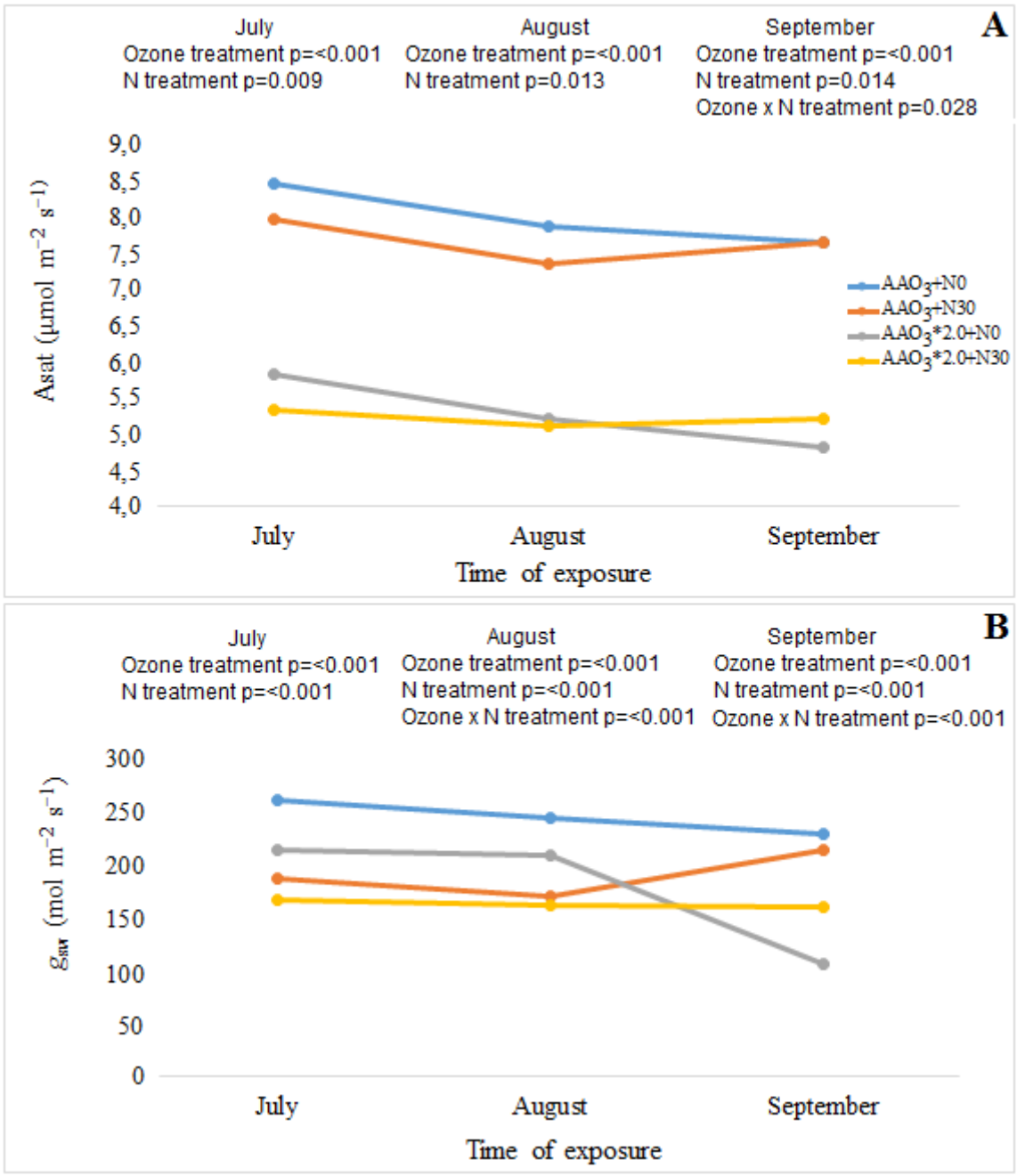

Fig. 3.3. Monthly average values (n = 3) of A_{sat} (light-saturated net photosynthetic rate) and g_{sw} (stomatal conductance for water vapour) in *Passiflora edulis* plants exposed to two ozone levels and two N doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30). *p* values of two-way ANOVA for factor 1 (Ozone treatments) and factor 2 (N treatments) and for factor 1 x 2 (when significant) were included on the top of each graph.

3.2.2. Photosystem II efficiency

F_o and F_m did not differ between O_3 and N treatments. However, significantly lower values of F_v/F_m were measured in plants exposed to AAO_3*2.0 than in plants grown under ambient O_3, regardless of the level of N fertilization (Table 3.2).

Table 3.2. Measurements of F_o, F_{max}, and F_v/F_m (Photosystem II efficiency) by Handy-PEA fluorimeter in *Passiflora edulis* exposed to two ozone levels and two N doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30) for 94 days. Data are shown as mean (n = 3). Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	F_o	F_m	F_v/F_m
AAO_3+N0	229 ± 15 Aa	853 ± 115 Aa	0.732 ± 0.02 Aa
AAO_3+N30	247 ± 14 Aa	937 ± 112 Aa	0.744 ± 0.03 Aa
AAO_3*2.0+N0	295 ± 21 Aa	802 ± 44 Aa	0.623 ± 0.03 Ab
AAO_3*2.0+N30	231 ± 33 Aa	926 ± 46 Aa	0.743 ± 0.04 Ab
	TWO WAY ANOVA (*p* values)		
O_3	0.350	0.681	**0.002**
N	0.391	0.175	0.131
O_3 x N	0.136	0.792	0.135

3.2.3. Growth and Biomass

The root and leaf biomass, root to shoot ratio and height did not vary significantly between both O_3 and N treatments.

The stem biomass was statistically higher and the stem diameter was lower in plants exposed to elevated O_3, either fertilized and not with N (AAO_3*2.0+N0; AAO_3*2.0+N30), than in plants exposed to ambient O_3 (AAO_3+N0; AAO_3*2.0+N30).

The leaf number increased in the plants exposed to AAO_3*2.0+N30 than in the AAO_3 plants, regardless the N supply they received and in plants fertilized with N compared to plants not fertilized with N, in both O_3 treatments (Table 3.3).

3.2.4 Total nitrogen, nitrate and ammonium

The N addition in soil (N30) increased the leaf concentrations of total N, NO_3^- and NH_4^+ in *P. edulis* compared to plants that were not fertilized with N, in both O_3 levels (±32% and ±33% respectively). Elevated O_3 also promoted a significant increase in the N and NO_3^- contents and a decrease in the NH_4^+ in plants supplied or not with N, in comparison to the leaf contents of plants exposed to ambient O_3 (Table 3.4)

Table 3.3. Leaf, stem, and root biomass (g), root/shoot ratio, height (cm), stem diameter (cm), and number of leaves of *Passiflora edulis* exposed to two ozone levels and two nitrogen doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30) for 94 days. Data are shown as mean ± S.E. Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Parameters	AAO_3 +N0	AAO_3 +N30	AAO_3*2.0 +N0	AAO_3*2.0 +N30	*TWO WAY ANOVA* (p values)		
					O_3	N	O_3 x N
Biomass							
Leaves	4.39 ± 0.20 Aa	5.30 ± 0.43 Aa	4.27 ± 0.42 Aa	4.42 ± 0.15 Aa	0.311	0.093	0.524
Stems	1.47 ± 0.15 Ab	1.73 ± 0.22 Ab	2.62 ± 0.07 Aa	2.92 ± 0.13 Aa	**<0.001**	0.075	0.886
Roots	1.11 ± 0.01 Aa	1.28 ± 0.16 Aa	1.12 ± 0.01 Aa	1.14 ± 0.01 Aa	0.225	0.075	0.208
Root/Shoot	0.62 ± 0.06 Aa	0.59 ± 0.05 Aa	0.50 ± 0.02 Aa	0.49 ± 0.06 Aa	0.070	0.750	0.883
Growth							
Height	72.9 ± 13.2 Aa	74.4±18.4 Aa	93.5±9.8 Aa	81.6 ± 13.6 Ab	0.271	0.440	0.939
Stem diameter	7.6 ± 0.4 Aa	8.4 ± 0.5 Aa	7.2 ± 0.5 Ab	6.5 ± 0.3 Ab	**0.032**	0.929	0.128
Leaf number	11.5 ± 2.3 Bb	16.5 ± 2.8 Ab	19.8 ± 2.2 Ba	24.6 ± 2.1 Aa	**0.029**	**0.013**	0.623

Table 3.4. Mean values of leaf nitrogen, nitrate (NO_3^-), and ammonium (NH_4^+) contents of *Passiflora edulis* plants exposed to two ozone levels and two nitrogen doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30). Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	N (mg g^{-1} DW)	NO_3^- (mg g^{-1} DW)	NH_4^+ (mg g^{-1} DW)
AAO_3+N0	12.97 ± Bb	22.3 ± 3.9 Ba	7.3 ± 1.8 Ba
AAO_3+N30	19.19 ± Ab	25.7 ± 3.6 Aa	20.4 ± 2.6 Aa
AAO_3*2.0+N0	14.35 ± Ba	25.2 ± 4.9 Bb	6.8 ± 1.7 Bb
AAO_3*2.0+N30	21.33 ± Ab	37.1 ± 5.9 Ab	13.7 ± Ab
	ANOVA test (p values)		
O_3	**<0.001**	**<0.001**	**0.021**
N	**<0.001**	**<0.001**	**0.001**
O_3 x N	0.919	0.743	0.621

3.2.5. Nitrate reductase and glutamine synthetase

Elevated O_3 (AAO_3*2.0) induced an increase in the nitrate reductase and a decrease in the glutamine synthetase of *P. edulis,* regardless level of N fertilization, compared to the enzymatic activities measured in plants grown under ambient O_3 (AAO_3). Nitrate reductase and glutamine synthetase activities were higher in *P. edulis* exposed N30 treatment than in plants exposed to N0 treatment, in both O_3 treatment (Table 3.5).

Table 3.5. Mean values of nitrate reductase and glutamine synthetase of *Passiflora edulis* plants exposed to two ozone levels and two nitrogen doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30). Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	Nitrate Reductase (μmol g^{-1} FW)	Glutamine Synthetase (μmol g^{-1} FW)
AAO_3+N0	21.02 ± 3.6 Bb	12.72 ± 2.3 Ba
AAO_3+N30	29.13 ± 3.7 Ab	18.31 ± 1.7 Aa
AAO_3*2.0+N0	26.50 ± 4.5 Ba	11.2 ± 1.4 Bb
AAO_3*2.0+N30	31.23 ± 4.9 Aa	15.7 ± 1.6 A
	ANOVA test (p values)	
O_3	**<0.001**	**<0.001**
N	**<0.001**	**<0.001**
O_3 x N	0.919	0.743

3.2.6. Pigments

Contents of chlorophyll *a* and *b* did not differ in plants exposed to both O_3 and N treatments. The leaf contents of carotenoids changed significantly only in response to elevated O_3, compared to the pigment level in plants exposed to AAO_3. It caused a reduction in the pigment of plants not fertilized and an increase in plants supplied with N (Table 3.6).

Table 3.6. Chlorophylls *a* and *b* (Chl *a*, *b*) and total carotenoids contents (mg mg^{-1} FW) in leaves of *Passiflora edulis* exposed to two ozone levels and two N doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30) for 94 days. Data are shown as mean (n = 3). Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	Chl *a*	Chl *b*	Carotenoids
AAO_3+N0	0.38 ± 0.02 Aa	0.17 ± 0.01 Aa	0.06 ± 0.01 Aa
AAO_3+N30	0.40 ± 0.02 Aa	0.18 ± 0.01 Aa	0.07 ± 0.02 Ab
AAO_3*2.0+N0	0.32 ± 0.02 Aa	0.22 ± 0.01 Aa	0.04 ± 0.01 Ab
AAO_3*2.0+N30	0.39 ± 0.03 Aa	0.24 ± 0.03 Aa	0.10 ± 0.01 Aa
	TWO WAY ANOVA (*p* values)		
O_3	0.419	0.138	**0.008**
N	0.142	0.807	0.221
O_3 x N	0.561	0.727	0.135

3.2.7. Proline and total flavonoids

The proline content increased in *P. edulis* leaves exposed to elevated O_3, regardless the N fertilization, compared to the levels measured in plants grown under AAO_3. The leaf content of proline did not change in response to N treatment, in both O_3 treatments (Table 3.7).

Leaf accumulation of total flavonoids was higher in plants exposed to AAO_3*2.0+N0 and to AAO_3*2.0+N30 than in plants from AAO_3. The content of flavonoids was higher in fertilized plants compared to unfertilized plants under AAO3 and lower under AAO_3*2.0 (Table 3.7).

Table. 3.7. Proline and flavonoids contents (mg mg^{-1} FW) in leaves of *Passiflora edulis* exposed to two ozone levels and two N doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30) for 94 days. Data are shown as mean ± S.E (n = 3). Lowercase letters indicate significant differences between ozone treatments and uppercase letters indicate significant differences between N treatments ($p < 0.05$, Holm-Sidak method).

Treatments	Proline (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
AAO_3+N0	0.39 ± 0.01 Ab	0.17 ± 0.01 Bb
AAO_3+N30	0.38 ± 0.02 Ab	0.22 ± 0.01 Ab
AAO_3*2.0+N0	0.53 ± 0.02 Aa	0.31 ± 0.01 Aa
AAO_3*2.0+N30	0.52 ± 0.02 Aa	0.24 ± 0.03 Ba
	TWO WAY ANOVA (*p* values)	
O_3	**<0.001**	**0.001**
N	0.391	**0.012**
O_3 x N	0.136	0.080

3.4. Metabolite profile

GC–EIMS analyses of *P. edulis* leaves revealed 27 major metabolites as follow: 9 carbohydrates (soluble sugars: sucrose, glucose, fructose, mannose, glucopyranose and sugar alcohols: arabitol, sorbitol, myo-inositol and mannitol), 3 fatty acids (palmitic, tetradecanoic and hexadecanoic acids), 6 organic acids (aconitic, isocitric, succinic, fumaric, malic and citric acids), ascorbic acid, 4 amino acids (serine, threonine, tyrosine and proline), 3 phenolic acids (benzoic, caffeic and shikimic acids) and 1 alkane (dodecane).

The leaf content of soluble sugars differed between O_3 and N treatments. Plants exposed in AAO_3*2.0+N0 and AAO_3*2.0+N30 showed higher contents of glucose and fructose. The profile of carbohydrates was influenced by the nitrogen levels applied to *P. edulis*; the concentration of sucrose, glucose and fructose registered was lower at AAO_3+N30 compared to AAO_3+N0. However, plants exposed in AAO_3+N30 showed higher contents of arabitol and glucopyranose.

Also, plants from AAO_3*2.0 treatments showed higher contents of sugar alcohols (*myo*-inositol and sorbitol), amino acids (threonine and proline), fatty acids (tetradecanoic and hexadecanoic acid), organic acids (citric, isotric and fumaric acids), and phenolic acids (caffeic acid) than plants exposed to AAO_3 treatment. The foliar content of ascorbic acid (AsA) increased significantly in plants exposed to AAO_3*2.0 treatments in relation to AAO_3 (Fig. 3.4).

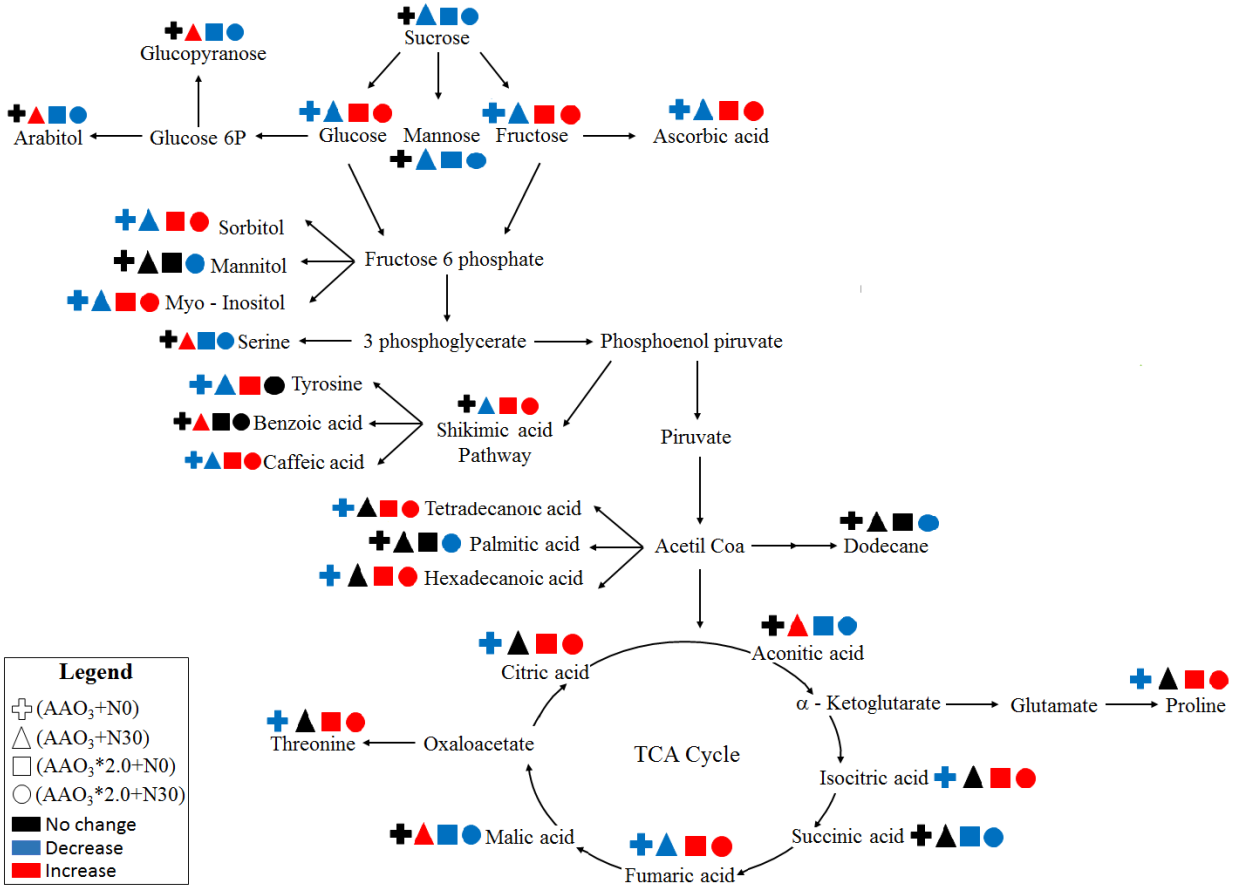

Fig. 3.4. Metabolite responses in *Passiflora edulis* plants exposed to two ozone levels and two nitrogen doses (AAO_3+N0; AAO_3+N30; AAO_3*2.0+N0; AAO_3*2.0+N30).

4. Discussion

Physio-biochemical adaptations of plants may have great importance in their growth, survival and yield under abiotic stress. Plants that are growing under natural conditions of pollutants and meteorological variables respond through various mechanisms of tolerance or sensitivity.

Thus, one of the most prominent effects of O_3 stress is the reduction in the gas exchange, resulting in decreases in plant growth and biomass production. In general, several authors reported that plants exposed to O_3 stress have lower stomatal conductance (g_{ws}) and light-saturated net photosynthetic rate (A_{sat}) (Singh *et al*., 2009; Matyssek *et al*., 2010; Hoshika *et al*., 2015; Yang *et al*., 2016; Chen *et al*., 2018), as found in our study. The monthly averages and overall average od A_{sat} and Ci/Ca ratio have been reduced in plants exposed in AAO3*2.0. However, nitrogen addition seemed to mitigate the effect of O_3 on gas exchange, since the plants that received nitrogen addition presented a softer decrease in A_{sat} (in both general and monthly average) and in general average Ci/Ca ratio, but showed an increase in monthly g_{sw} when exposed to elevated O_3 level. Similar results were found by Liu *et al*. (2020) in tropical and subtropical plants in China (*Ardisia quinquegona* and *Blastus cochinchinensis*), which received a canopy N addition of 25 kg N ha^{-1} yr^{-1} and 50 kg N ha^{-1} yr^{-1}, respectively. The A_{sat} was significantly higher in both plant species.

Several studies have found that N addition significantly enhanced the leaf photosynthetic rate, mainly because this element is an important component of chlorophyll, photosynthesis-related enzymes, and other proteins, promoting leaf development (Wang *et al*., 2018; Liang *et al*., 2020). N supply increased the stomatal conductance and other photosynthetic parameters in several other plant species (Wall *et al*., 2000; Chen *et al*., 2005; Yamaguchi *et al*., 2007; Mao *et al*., 2014; Zhang *et al*., 2018; Yuan *et al*., 2020). However, the metanalysis performed by Feng *et al*. (2019) indicated that the benefic effect of N fertilization was not enough to compensate the negative effect of elevated O_3 on photosynthetic rate in most trees, perennial or annual non-wood forbs and graminoids studied. The negative effect of elevated O_3 on stomatal conductance of these functional groups was also not altered by any levels of N addition. The low potential photochemical efficiency, verified by the reduced of F_v / F_m ratio, was only detected in non-fertilized plants of *P. edulis* grown under elevated O_3 (AAO_3*2.0 + N0 treatment). This suggests that O_3 may have caused PSII photoinhibition, considering that F_v/F_m was below the range ($0.80 \leq F_v/F_m \leq 0.86$) reported by Björkman & Demmig (1987) for healthy plants (Peng *et al*., 2019). Some authors suggest that O_3 can cause oxidative damage to the photochemical apparatus (Calatayud *et al*., 2002) and inactivate PSII D1 protein (Guidi & Degl'Innocenti, 2008; Pellegrini

et al., 2011). However, the reduction of F_v/F_m ratio was not evidenced in the fertilized plants growing under elevated O_3 (AAO_3*2.0 + N30 treatment). This result highlights again that the plant fertilization with N alleviated the oxidative effects of O_3 on the photosynthetic apparatus of the *P. edulis* plants. Opposite results were found by Calatayud *et al*. (2006) for *Citrillus lanatus* that demonstrated the interaction between ozone and N led to a significant decrease in maximum efficiency of PSII photochemistry (F_v/F_m) and induced a significant decrease in the actual quantum yield of PSII.

In relation to leaf development, plants can produce new leaves to compensate low photosynthetic rates and support the mobilization of reserves. The number of leaves was increased in plants exposed to AAO_3*2.0+N30, as also observed by Moura *et al*. (2018) in the sugarcane genotype IACSP95-5000, Engela *et al*. (2020 submitted) in *Eugenia uniflora*, and by Hoshika *et al*. (2020) in *Alnus glutinosa* exposed to different ozone levels. This result may indicate a compensation to recover photosynthetic rates and the balance between production rate of new leaves and abscission rates of old leaves (Hikosaka, 2004; Shimizu *et al*., 2006; Falster *et al*., 2018; Grulke & Heath, 2019). The N fertilization also stimulated the leaf production in the *P. edulis* plants exposed either to ambient or elevated O_3, pointing to an additive and positive effect of both pollutants (Schmutz *et al*., 1995). New leaves promote longitudinal growth and play an important role in the growth and development of the plant (Zhang *et al*., 2020).

The weak interacting effect of both O_3 and N supply on plant height corroborates the findings of most studies included in the revision presented by Feng *et al*. (2019). However, contradicting these studies, the growth in stem and biomass diameter of *P. edulis* plants was stimulated by O_3 in the liana species studied, although a positive nutritional effect of the N supply was expected. At least in trees, the N storage is seasonally programmed, closely linked to tree phenology and operates at temporal scales of months to years, with remobilization being source driven (Millard *et al*., 2006; Millard *et al*., 2007). Also, trees accumulate large amounts of C as non-structural carbohydrates (NSC) in throughout the plant, including in leaves and stems (Hoch *et al*., 2003; Würth *et al*., 2005; Palacio *et al*., 2008; Spann *et al*., 2008; Macieira *et al*., 2020). Although the concentration is larger in the phloem, the nonstructural carbon reserves are considerably larger in the wood due to the greater mass proportion of this tissue (Würth *et al*., 2005; Gruber *et al*., 2014; Locoselli & Buckeridge, 2017).

Natural pigments in plants are responsible for essential functions in vegetable cells as protection and metabolic reactions (Aguirre-Joya *et al*., 2020). In plants, for example, chlorophylls are the most abundant pigments and their role in photosynthesis is vital. Chlorophylls are located in chloroplasts and driving photosynthesis by absorbing light and transducing it into

chemical energy (Agathokleous *et al*., 2020). In our study, we found that Chlorophyll *a* and *b* contents did not differ in plants exposed to both O_3 and N treatments. Several studies show that contents of chlorophyll *a* reduce in leaves by different O_3 levels (Wang *et al*., 2020) and increase by N fertilization (Prsa *et al*., 2007; Bassi *et al*., 2018). Although no statistically significant different, plants exposed to high ozone and that received nitrogen addition showed 18% more chlorophyll *a*. Thus, we can believe that the addition of nitrogen seemed to alleviate the effects of O_3.

Another important class of pigments in plants are carotenoids; they 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; Genç *et al*., 2020). Carotenoid content showed opposite trend than chlorophyll *a* in leaves of *P. edulis*. The accumulation of carotenoids in plants under high O_3 could play a role in the protection of *P. edulis* plants from oxidative damage (MirAafaq *et al*., 2013).

Nitrogen is important for the synthesis of amino acids, proteins, chlorophylls, nucleic acids, and lipids (Calatayud *et al*., 2007; Kusano *et al*., 2011). Nitrate (NO_3^-) and ammonium (NH_4^+) are the main sources of inorganic nitrogen for plant growth. The leaf capacity of N assimilation is associated with activities of primary nitrogen assimilation pathway enzymes, in plants, NO_3^- is converted into nitrite (NO_2^-) by nitrate reductase in the cytosol. NO_2^- can be further reduced to NH_4^+ via nitrite reductase (NiR) in chloroplasts or plastids. NH_4^+, derived either from NO_3^- reduction or from direct uptake, is first converted to glutamine (Gln) by glutamine synthetase (GS) and then to glutamate (Glu) by glutamate synthase (GOGAT) (Foyer *et al*., 2006; Sanchez-Rodriguez *et al*., 2011; Liu *et al*., 2014). In our study, it was possible to verify that both O_3 and N supply affected positively the contents of total N and NO_3^-, and the NR activity, demonstrating that N assimilated as NO_3^- was converted into NO_2^-in plants of all treatments. However, opposite effects of O_3 and N supply were observed in the leaf concentrations of NH_4^+ and GS activity. While the N supply increased both parameters, the elevated O_3 decreased them. This means that elevated O_3 seemed to restrict N metabolism of *P. edulis* in the phase of the NO_2^- reduction to NH_4^+ catalyzed by GS. This finding may explain the NO_3^- accumulation and consequent no restrictions on the chlorophyll levels and growth and biomass production in the plants exposed to elevated O_3, despite the photosynthetic reductions. We may consider that NO_3^- and NO_2^- (not quantified in the present study) are essential for plant growth according to van den Berg & Ashmore (2008). In addition, the plants exposed to AAO3*2.0 were exposed to a lower toxicity level induced by NH_4^+ compared to plants exposed to ambient O_3. It is known that elevated NH_4^+ uptake decreases photosynthetic capacity and results in cation deficiency and

chlorosis (Van den Berg & Ashmore, 2008). Summarizing, the high doses of O_3 appeared to affect the N assimilation by plants in a beneficial way.

O_3 and N treatments can decrease, increase, or have no effect in the metabolite profile of plants, depending on different cultivars and varieties, concentration and exposure time. In the present study, elevated O_3 concentration increased sugar alcohols, amino acids, fatty acids, organic acids, and phenolic acids. However, the carbohydrate profile was influenced by the N addition; the plants from the AAO_3+N30 treatment showed the lowest values of soluble carbohydrates. This suggests that when nitrogen is not a limiting factor, the translocation of carbohydrates from leaves to other plant parts, such as root for example, increases. In this sense, Wang *et al*. (2018) suggest that carbon and nitrogen metabolism are tightly coordinated in the fundamental biochemical pathway in plants. Li *et al*. (2012) also indicated that increased N supply leads to a significant decrease in photosynthetic N use efficiency. Liu *et al*. (2016) in their meta-analysis demonstrated that N addition may also damage the photosynthetic system and increase energy and C skeletons consumption due to N assimilation.

The N addition can also increase the organic acids. Sun *et al*. (2020) showed increases in organic acids involved in the TCA cycle with increased NO_3^- content and Segade *et al*. (2017) showed increases in citric acid in O_3-treated grapes. In our study, we found that plants exposed to high O_3 and that received N fertilization had higher concentrations of citric, fumaric and isocitric acids.

Flavonoids are structurally diverse secondary metabolites and have long been suggested as performing multiple function protections in plants. Major roles of flavonoids in plants include antioxidant and anti-radical functions, scavenging of ROS in plant tissues, provide colors to flowers and other parts, and modulates the auxins transport (Agati *et al*., 2012; Davies *et al*., 2012; Brunetti *et al*., 2013). In our study, content of flavonoids was higher in plants exposed to high levels of O_3. According to some authors, plants under O_3 stress increase the concentrations of phenolic compounds, including flavonoids (Saviranta *et al*., 2010; Engela *et al*., 2020 submitted). Thus, we believe that the IAC 273/277 genotype of *P. edulis* presents more activated defense responses under O_3 pollution. However, the effect of N supply on this defense response was dependent on the level of O_3 exposure. It was stimulated only in plants submitted to ambient O_3.

The increased content of ascorbic acid also detached in the metabolite profile analysis could contribute to explain the good adaptability of *P. edulis* to high levels of O_3 and N pollution. Fernandes *et al*. (2019) demonstrated that plants *Passiflora edulis* also showed high values of ascorbic acid after the exposure to high O_3. These results confirm that ascorbic acid play an

important role in the protection of this liana species against O_3, as observed in many other studies (e.g. Noctor & Foyer, 1998; Chen & Gallie, 2005; Frei *et al*., 2012; Podda *et al*., 2019).

Under the influence of stress, the metabolism of amino acids is significantly modified, reducing the synthesis of proteins and inducing their rapid degradation, leading to an increase in amino acids and free amines, such as an increase in proline synthesis (Silveira *et al*., 2002; Sodek, 2004). The increase in proline amounts, as detected in *P. edulis* plants either N fertilized or not and grown under high O_3 levels, can stimulate different cellular functions, including osmotic adjustment, carbon and nitrogen reserve for growth and recovery after stress, detoxification of excess of ammonia, stabilization of proteins and membranes, and elimination of free radicals (Paulus *et al*., 2010). Amino acids can also act as stress-reducing agents, source of nitrogen, blocks of proteins, transporter of organic nitrogen through the plant, and hormone precursors (DeLille *et al*., 2011; Maeda & Dudareva, 2012). In this sense, glutamate can act to attenuate oxidative stress indirectly because it is a precursor to other amino acids such as arginine and proline, which are related to the reduction of plant stress (Gill & Tuteja, 2010; Rejeb *et al*., 2014).

Regarding metabolites, plants synthesize L-proline in the cytosol in response to various environmental stresses. L-Proline accumulation in plants facilitates water uptake and have several protective functions, such as osmoprotectant, stabilizer of cellular structures and enzymes (Székely *et al*., 2008; Meena *et al*., 2019). Proline protects the cell against ROS accumulation under stress conditions by recycling of NADPH via its synthesis from glutamate and by acting as a free radical scavenger (Soares et *al*., 2018). Signorelli *et al.* (2013) proposed a proline cycle that may act in scavenging ·OH. In our study, the significant increase of proline that occurred in plants exposed in AAO_3*2.0 indicates that this amino acid was involved in a direct antioxidative protection or served as ascorbic acid regeneration to maintain the cellular redox potential (Zhang & Becker, 2015). In this sense, the N supply did not alter the efficiency of proline as a non-enzymatic antioxidant against ROS.

5. Conclusions

The whole set of results led us to accept the proposed hypothesis and conclude that N deposition on soil alleviates the O_3 effects in *P. edulis*, opposing the tendencies observed in plant species belonged to other functional groups already studied. In present study, the N supply alleviated the effects of elevated O_3 levels on growth, biomass, photosynthesis, and chlorophyll fluorescence, but also elevated the antioxidative defense components analyzed in *P. edulis*.

The elevated O_3 affected the N metabolism of *P. edulis* in a beneficial way. The phase of the nitrite reduction to ammonium catalyzed by glutamine synthetase was restricted, resulting in NO_3^- accumulation and consequent no alterations in the chlorophyll levels and growth and biomass production in the plants, despite the photosynthetic reductions. In addition, the plants exposed to high O_3 levels were exposed to a lower secondary stress induced by NH_4^+.

The absence of visible foliar injury, non-reductions in growth and biomass production and significant increases in fatty acids, sugar alcohols, amino acids (such as proline), ascorbic acid, and flavonoids contents, and the increased number of leaves, all together suggest that the cultivar of *Passiflora edulis* studied is able to tolerate the oxidative stress induced by the interactive effects of O_3 and nitrogen addition on soil.

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Conclusões Finais

Os resultados obtidos e apresentados nos três capítulos anteriores permitiram validar duas hipóteses formuladas na presente tese:

(1) As espécies arbóreas pioneiras e lianas são menos susceptíveis ao estresse oxidativo induzido por fatores de estresse ambiental de origem natural e antrópica do que as espécies não pioneiras, considerando que as primeiras estão adaptadas à neutralização de níveis basais mais altos de ERO decorrentes de processos fisiológicos naturais, como fotossíntese e respiração.

(2) Maior diversidade metabólica ou níveis mais altos de alguns grupos de metabólitos podem incrementar a tolerância das espécies nativas frente aos fatores de estresse ambiental.

No presente estudo, a utilização da abordagem funcional ecossistêmica ajudou no entendimento da plasticidade de respostas das plantas aos diversos estressores a que estão submetidas nos remanescentes florestais de Mata Atlântica do sudeste brasileiro. Dentre os três grandes grupos funcionais estudados: as espécies arbóreas pioneiras e lianas parecem ser mais tolerantes ao estresse oxidativo e, portanto, possuem maior capacidade de dominar fragmentos perturbados por ações antrópicas. Por outro lado, as espécies não pioneiras são suscetíveis ao estresse oxidativo e apresentam baixa plasticidade de resposta a mudanças ambientais e menor capacidade de dominar áreas perturbadas. Finalmente, esta tese ressalta a relevância das características fisiológicas, bioquímicas, padrões de crescimento e alterações metabólicas para o entendimento das estratégias de aclimatação/adaptação ao estresse oxidativo dos diferentes grupos funcionais de plantas, principalmente aos estresses combinados de ozônio e adição de nitrogênio no solo em lianas, nunca estudado anteriormente.

Os resultados incluídos nos três capítulos evidenciam, assim, a importância da associação de estudos em nível experimental planejado e em nível ecossistêmico para avaliação da capacidade de aclimatação de espécies nativas da Floresta Atlântica aos estressores ambientais.