UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Avaliação dos efeitos do ozônio sobre parâmetros bioquímicos e fisiológicos de folhas e frutos de *Capsicum baccatum* L. var. *pendulum*

RAFAEL CALIXTO BORTOLIN

Orientador
Dr. JOSÉ CLÁUDIO FONSECA MOREIRA

- Porto Alegre -
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"Somos todos geniais.

Mas se você julgar um peixe por sua capacidade de subir em árvores, ele passará sua vida inteira acreditando ser estúpido."

Albert Einstein

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LISTA DE ABREVIATURAS

APX - ascorbato peroxidase
CAT - catalase
ERO - espécies reativas de oxigênio
eV - electron volt
GPX - glutationa peroxidase
H_2O_2 - peróxido de hidrogênio
HPLC - cromatografia líquida de alta eficiência do inglês: High-performance liquid chromatography
$O_2^{\bullet-}$ - superóxido
$OH^{\bullet}$ - radical hidroxil
O_2 - oxigênio molecular
O_3 - ozônio
ppb - partes por bilhão
SOD - superóxido dismutase

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RESUMO

O ozônio (O_3) é um potente agente oxidante capaz de reagir com várias biomacromoléculas. O O_3 troposférico é o poluente atmosférico mais danoso às culturas vegetais, contribuindo para grandes perdas da produtividade agrícola. As concentrações troposféricas de O_3 vêm aumentando desde os tempos pré-industriais atingindo concentrações fitotóxicas em várias regiões do mundo. As pimentas do gênero *Capsicum* são o segundo tempero mais comercializado no mundo, mas poucos estudos em relação aos efeitos do O_3 neste gênero são conhecidos. Portanto, o objetivo deste trabalho é avaliar os efeitos da exposição crônica a altas concentrações de O_3 em plantas da espécie *Capsicum baccatum* L. var. *pendulum*, principalmente sobre o estado redox do tecido foliar, bem como produtividade e qualidade de seus frutos. Para isso, quinze plantas da espécie *C. baccatum* foram expostas ao O_3, em câmeras de topo aberto durante o período de amadurecimento do fruto (62 dias), sob uma concentração média de 171.6 $\mu g/m^3$ das 10:00 as 16:00. Neste trabalho, mostramos que o O_3 desencadeou uma série de alterações deletérias no tecido foliar (lipoperoxidação e carbonilação protéica aumentada, aumento nos níveis de espécies reativas de oxigênio (ERO), degradação aumentada de clorofila, entre outros) de plantas *C. baccatum* L. var. *pendulum*, quando estas foram expostas cronicamente a altos níveis deste poluente. Além disso, observamos que estas alterações se estenderam aos frutos, culminando em diminuição da produtividade e modificação da composição química dos mesmos (diminuição nos níveis de capsaicina, aumento nos níveis de carotenóides e polifenóis totais). Esta alteração na composição química de frutos ozonizados se refletiu negativamente sobre o potencial antioxidante dos mesmos. Portanto, a exposição ao ozônio alterou a qualidade do fruto; porém testes *in vivo* são necessários para definir o impacto destas alterações no potencial terapêutico da pimenta.

ABSTRACT

Ozone (O_3) is a powerful oxidizing agent capable of reacting with several biomacromolecules. Tropospheric O_3 is one of the most harmful air pollutants to crops, contributing to high losses on crop yield. Tropospheric O_3 background concentrations have increased since pre-industrial times reaching phytotoxic concentrations in many world regions. *Capsicum* peppers are the second most traded spice in the world, but few studies concerning the O_3 effects in this genus are known. Thereby, the aim of this work was to evaluate the effects of chronic exposure to elevated O_3 concentrations in red pepper plant *Capsicum baccatum* L. var. *pendulum* with especial considerations on the leaf redox state, as well as fruit yield and quality. Fifteen *C. baccatum* plants were exposed to O_3 in open-top chambers during fruit ripening (62 days) at a mean concentration of 171.6 μg/m^3 from 10:00 am to 4:00 pm. In this study we show that the O_3 triggered a series of deleterious changes in leaf tissue (increased lipid peroxidation and protein carbonylation, increased ROS levels, increased chlorophyll degradation, etc.) of *C. baccatum* L. var. *pendulum*, when they were chronically exposed to high levels of this pollutant. Furthermore, we observed that these changes were extended to the fruits, resulting in decreased productivity and modification of the chemical composition (capsaicin levels diminished, increased total carotenoids and polyphenols levels). These chemical composition changes on fruits of ozonated plants were negatively reflected on their antioxidant potential. In conclusion, O_3 exposure altered fruit quality, but *in vivo* tests are necessary to define the impact of these changes on the pepper therapeutic potential.

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1. INTRODUÇÃO

1.1. Ozônio

1.1.1. Visão geral

O aumento na proporção de diversos poluentes na atmosfera vem representando uma grande ameaça aos mais diversos ecossistemas mundiais, bem como à saúde humana e produtividade agrícola. Na maioria das vezes, estes poluentes tem sua origem em distintas atividades humanas, e a concentração dos mesmos cresce cada dia mais devido, principalmente, ao aumento populacional e crescente industrialização (IPCC, 2001; Fuhrer, 2009; Cho et al., 2011).

Atualmente, entre os poluentes atmosféricos, o ozônio (O_3) tem sido considerado, por alguns autores, um dos poluentes mais prejudiciais às culturas agrícolas levando a grandes perdas econômicas (Heagle, 1989; Ashmore, 2005). Sua concentração tem aumentado muito desde os tempos pré-industriais e projeções mostram que irá aumentar ainda mais principalmente em países em desenvolvimento (IPCC, 2001).

Descobertas recentes têm mostrado que o O_3 não afeta apenas a produtividade das plantas, mas também a qualidade final do produto agrícola por alterar seus componentes (Ashmore, 2005). Consequentemente, estas mudanças na composição química podem trazer prejuízos à segurança alimentar e saúde do consumidor como discutido por Vandermeiren e Pleije (2011). Devido à importância e recente preocupação com a qualidade dos alimentos, a alteração da qualidade de produtos agrícolas causada pelo O_3 tem impulsionando novas pesquisas na área de qualidade alimentar (Wang e Frei, 2011).

1.1.2. Ocorrência e formação

O O_3 é um gás presente naturalmente tanto na estratosfera quanto na troposfera. Na estratosfera, tem um papel de filtragem dos raios ultravioleta e forma-se a partir da fotodissociação do oxigênio molecular (O_2) em dois átomos de oxigênio que posteriormente se combinam com outro O_2 para formar o O_3 (Equação 1). Na troposfera, camada na qual estamos, o O_3 forma-se a partir de uma série de -reações complexas envolvendo luz solar, O_2 e poluentes primários, como óxidos de nitrogênio, óxidos de enxofre, óxidos de carbono e hidrocarbonetos (Equações 2 e 3 como exemplo). Nesta camada ele exerce um efeito nocivo aos organismos vivos devido ao seu alto poder oxidativo (+2.07 eV) (Crutzen e Lelieveld, 2001; IPCC, 2001). Atualmente, as principais fontes de poluentes primários são derivadas da atividade humana, tais como, queima de combustíveis fósseis, processos industriais, queimadas florestais e decomposição de resíduos agrícolas (Fowler et al., 1999; Crutzen e Lelieveld, 2001).

Equação 1: $O_2 + h\nu \rightarrow 2O \quad (\lambda \leq 240\,nm) \longrightarrow O + O_2 + M \rightarrow O_3 + M(2x)$

Equação 2: $NO_2 + h\nu \rightarrow NO + O \quad (\lambda \leq 400\,nm) \longrightarrow O + O_2 + M \rightarrow O_3 + M$

Equação 3: $CH_4 + 8O_2 \rightarrow CO + H_2O + 4O_3 + 2HO,$

1.1.3. Concentrações troposféricas: passado, presente e futuro

Devido à emissão destes poluentes primários, a concentração de ozônio troposférico aumentou 36% desde os tempos pré-industriais e projeções mostram que a concentração atual tende a aumentar ainda mais (variando de 42 a 84 ppb para o final do século), principalmente em países em

desenvolvimento decorrente da rápida industrialização (IPCC, 2001; Vingarzan, 2004; Karnosky et al., 2007; Avnery et al., 2011a).

No Brasil as concentrações de ozônio vêm aumentando principalmente em grandes centros urbanos como São Paulo e Porto Alegre, onde excede, várias vezes ao ano, o limite estabelecido pelo conselho nacional do meio ambiente (CONAMA) de 160 $\mu g.m^3$ (~80 ppb), em monitoramento contínuo de 1 hora (Petrobras, 1998; Cetesb, 2009).

1.1.4. Potencial oxidativo: ação em organismos vivos

O O_3 é um forte agente oxidante capaz de reagir com lipídios, proteínas, ácidos nucléicos e carboidratos quando absorvido por animais e plantas, além de favorecer a formação de espécies reativas de oxigênio (ERO), tais como peróxido de hidrogênio (H_2O_2), superóxido ($O_2^{\bullet -}$) e radical hidroxil ($OH^{\bullet}$). Quando os níveis de ERO excedem a capacidade de defesa da célula temos um quadro caracterizado como estresse oxidativo. Quando estabelecido este quadro, podem ocorrer danos oxidativo às biomoléculas e alteração no perfil oxidativo das células, assim, levando a uma série de problemas relacionados tanto a saúde humana quanto à produção agrícola (Iriti e Faoro, 2008; Sharma et al., 2012).

1.2. Ação do ozônio nas plantas

1.2.1. Fitotoxicidade

Em plantas, o O_3 penetra nas folhas através dos estômatos, onde é dissolvido no fluido apoplástico. Uma vez dentro da câmara substomatal, o O_3 pode ser espontaneamente decomposto em ERO ou reagir com vários compostos presentes na parede celular, fluido apoplástico e membrana

plasmática para formar ERO. Pela alta reatividade do O_3, o mesmo não consegue se difundir muito além da câmara substomatal, ou seja, seus efeitos danosos são geralmente ligados às ERO geradas por ele (Castagna e Ranieri, 2009). A exposição ao O_3, seja crônica ou aguda, possui efeitos adversos as plantas, que geralmente apresentam danos visíveis principalmente nas folhas. Os sintomas visíveis mais comuns devido à exposição crônica ao O_3 são clorose (pontos amarelados devido à degradação da clorofila) e o aparecimento de pontos marrom-avermelhados devido ao acúmulo de fenilpropanóides. Por outro lado, a exposição aguda tem efeitos muito mais diversos, alguns deles são: manchas esbranquiçadas (um tipo de necrose com ausência de pigmentação), manchas marrons (um tipo de necrose com presença de pigmentação) e pequenos pontos que variam entre branco, vermelho e preto (Figura 1).

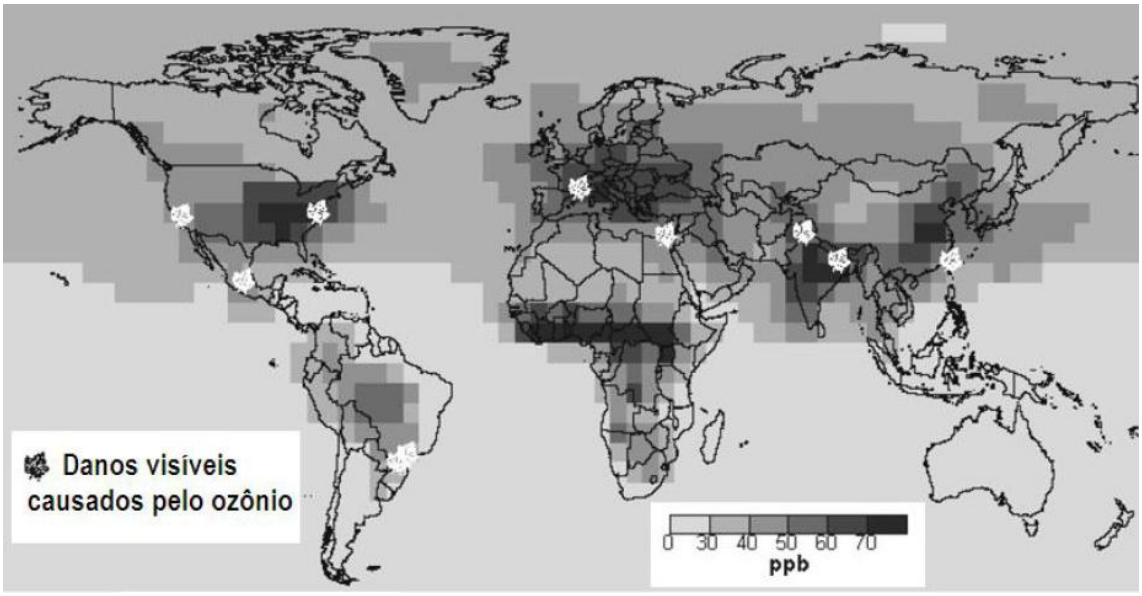

Figura 1: O mapa mostra as localidades ao redor do mundo onde já se tinha evidências em 2003 de danos visíveis ou prejuízo na produtividade devido ao ozônio (símbolo em forma de folha), além de apresentar a distribuição global da concentração média de ozônio para o ano de 1999. Modificado a partir de Ashmore (2005).

Plantas expostas ao O_3 nem sempre manifestam danos visíveis, muitas vezes elas sofrem com danos invisíveis, que incluem redução da fotossíntese, perda de água, esterilidade do pólen, biomassa e produtividade reduzida (Iriti e Faoro, 2008; Cho et al., 2011; Wilkinson et al., 2012).

1.2.2. Prejuízos a agricultura

Além dos efeitos deletérios sobre o metabolismo de plantas, muitos estudos têm discutido também as perdas econômicas. O O_3 troposférico tem sido considerado, por alguns autores, o poluente atmosférico mais prejudicial às culturas agrícolas levando a grandes perdas na economia (Heagle, 1989; Ashmore, 2005). Espécies sensíveis ao O_3, como melancia e tomate, podem chegar a perdas entre 17 e 39 % da produção em alguns lugares do mundo (Cho et al. 2011). De acordo com Avnery e colaboradores (2011b) para o ano de 2000 as perdas globais na produção de soja, trigo e milho devido ao O_3 variaram entre 8.5–14%, 3.9–15% e 2.2–5.5%, respectivamente. Para o mesmo ano a perda na produtividade mundial devido ao O_3 foi estimada em 79-121 milhões de toneladas contabilizando US$ 11–18 bilhões (Avnery et al., 2011b). Outros estudos mostram que estas perdas podem ser ainda maiores chegando a US$ 26 bilhões e que 40 % deste prejuízo ocorrem na Índia e na China, principalmente devido ao seu rápido crescimento econômico e industrialização (Ashmore, 2005; Van Dingenen et al., 2009).

1.2.3. Mecanismos de defesa nas plantas

As ERO são subprodutos normais do metabolismo celular, porém quando são produzidas de forma exacerbada e desregulada podem danificar estruturas celulares. Para evitar estes danos, as plantas desenvolveram um

eficiente sistema de defesa, o qual é responsável por manter as ERO a níveis basais (Halliwell e Gutteridge, 2007; Castagna e Ranieri, 2009). Um dos primeiros mecanismos de defesa contra a entrada de gases tóxicos, inclusive O_3, é o fechamento dos estômatos, muitas vezes sinalizadas pelo aumento nas ERO (Iriti e Faoro, 2008; Sharma et al., 2012). Além deste sistema, as plantas apresentam um sistema antioxidante enzimático e outro não enzimático.

As defesas antioxidantes enzimáticas, em plantas, incluem superóxido dismutase (SOD), ascorbato peroxidase (APX), glutationa peroxidase (GPX) e catalase (CAT). A SOD, encontrada em quase todos os compartimentos, atua como a primeira linha de defesa contra ERO dismutando $O_2^{\bullet-}$ em H_2O_2, enquanto as enzimas APX, GPX e CAT atuam subsequentemente detoxificando H_2O_2 em produtos inofensivos. A CAT é principalmente encontrada nos peroxissomos e converte H_2O_2 em O_2 e água, enquanto APX é a principal peroxidase operando nos cloroplastos. Além destas enzimas, as células possuem uma grande quantidade de compostos antioxidantes não-enzimáticos com um alto poder redutor que também são responsáveis pela detoxificação das ERO, como por exemplo, glutationa, tocoferol, ácido ascórbico e uma variedade enorme de compostos fenólicos (Noctor e Foyer, 1998; Blokhina et al., 2003; Halliwell e Gutteridge, 2007; Gill e Tuteja, 2010; Sharma et al., 2012).

1.2.4. Troca de metabolismo e alteração na qualidade da produção

Muitos compostos secundários além de serem antioxidantes podem apresentar outras propriedades importantes para as plantas, tais como potencial antimicrobiano, anti-herbivoria e proteção contra a radiação ultravioleta, e por isso são componentes chave do sistema de defesa das

plantas atuando na proteção contra uma variedade situações de estresse, tais como herbivoria, infecção microbiana, exposição ao ozônio, luz ultravioleta e seca. A geração aumentada de ERO nestas situações pode levar a dois efeitos opostos: 1) ser responsável pelo efeito prejudicial (quando as ERO ultrapassam a capacidade dos mecanismos de defesa antioxidante) ou 2) servir como sinalizador para ativar mecanismos de tolerância ao estresse, por exemplo, aumentando a produção de metabólitos secundários. Geralmente, compostos secundários dependem dos compostos primários como fonte de precursores. Portanto, situações de estresse (por exemplo, exposição ao O_3) podem causar uma troca de metabolismo, desviando recursos disponíveis ao metabolismo de crescimento para o metabolismo de defesa, assim podendo levar a uma redução no crescimento, bem como, alterações na composição química das plantas e seus frutos, consequentemente alterando a qualidade dos produtos agrícolas (Korkina, 2007; Mattson, 2008; Iriti e Faoro, 2009; Sharma et al., 2012). Além disso, devido à grande atividade biológica desses compostos, membros destes grupos podem fornecer benefícios à saúde e/ou ser tóxicos para humanos, mostrando a importância de avaliar estes compostos como parâmetros de qualidade em produtos agrícolas.

1.2.5. Qualidade dos produtos agrícolas

O ozônio leva a prejuízos a agricultura pela diminuição da produtividade o que ameaça a segurança alimentar principalmente em países em desenvolvimento onde a falta de alimentos já é um problema. Porém, o O_3 não ameaça a agricultura e segurança alimentar somente por reduzir a quantidade de alimentos, mas também por alterar sua qualidade. Porém, muito mais atenção tem sido dada aos impactos do O_3 sobre a produtividade, devido a

interesses econômicos, do que a qualidade da produção (Vandermeiren e Pleije, 2011).

A qualidade dos produtos agrícolas pode ser afetada tanto por uma mudança no metabolismo primário das plantas cultivadas (por exemplo, carboidratos, lipídios e proteínas), ou por uma alteração em seus compostos secundários (tais como, compostos fenólicos, carotenóides e alcalóides). Dentre os poucos estudos que se tem avaliando a qualidade dos alimentos, a maioria deles ficam restritas as alterações no conteúdo de carboidratos, lipídios e proteínas, ou seja, quase não há estudos sobre alterações nos compostos secundários das plantas. Porém estes compostos determinam inúmeros aspectos ligados à qualidade dos alimentos, tais como cor, sabor, aroma e potenciais terapêuticos (ver seção 1.5. Referencial Teórico).

1.3. Compostos secundários e saúde humana

Diversos fitoquímicos derivados do metabolismo de defesa (metabolismo secundário) são importantes para a saúde e nutrição humana, especialmente porque muitos deles são fontes de substâncias biologicamente ativas. Muitas destes compostos apresentam propriedades benéficas à saúde, tais como potencial anticâncer, anti-inflamatório e antioxidante; porém outros podem ser prejudiciais a saúde apresentando toxicidade e potencial carcinogênico (Chen e Kong, 2004; Crozier et al., 2006; Korkina, 2007).

Estudos epidemiológicos confirmam que uma dieta composta de alimentos ricos em polifenóis está associada com a prevenção de várias patologias. Estes resultados poderiam ser justificados pelos efeitos antioxidantes e anti-inflamatório descritos para muitos fitoquímicos como

fenólicos, carotenóides e capsaicinóides; uma vez que inflamação crônica e o desbalanço oxidativo são os principais determinantes para o aparecimento de doenças degenerativas, tais como câncer, doenças cardiovasculares, diabetes tipo 2 e as várias neuropatias (Pietta, 2000; Scalbert et al., 2005; Hounsome et al., 2008; Kang et al., 2010).

1.4. Gênero Capsicum

1.4.1. Visão geral

O gênero *Capsicum* é nativo da América Latina e compreende cerca de 30 espécies diferentes das quais apenas cinco são domesticadas e comumente cultivadas: *Capsicum annuum* L., *Capsicum chinense Jacq*., *Capsicum frutescens* L., *Capsicum baccatum* L., e *Capsicum pubescens* (Bosland e Votava, 2012; Wahyuni et al., 2013). Os frutos das plantas deste gênero, comumente conhecidas como pimentas vermelhas, são usados no mundo inteiro como temperos para dar cor, realçar o sabor e fornecer pungência à comida (De, 2003). Além disso, estes frutos apresentam uma série de compostos benéficos à saúde, tais como vitaminas, carotenóides, compostos fenólicos e capsaicinóides (Edge et al., 1997; Rice-Evans et al., 1997; Oyagbemi et al., 2010; Wahyuni et al., 2011). Dentre os capsaicinóides, metabólitos pungentes que são característicos do gênero *Capsicum*, a capsaicina (8-metil-N-vanilil-6-nonenamida) é o mais bem estudado, principalmente por apresentar importantes propriedades farmacêuticas, tais como potencial anti-inflamatório, antioxidante, antiproliferativo e anticâncer (Hoffman et al., 1983; Oyagbemi et al., 2010). As pimentas vermelhas são o segundo tempero mais comercializado no mundo, perdendo apenas para as

pimentas do gênero *Piper* (pimentas do reino), e sua produção mundial tem aumentado substancialmente nos últimos anos (Weiss, 2002; Bosland e Votava, 2012). Apesar de serem nativas da América, os maiores consumidores e produtores das pimentas *Capsicum* são China, India e México, os quais são países em desenvolvimento, onde é previsto que as concentrações de O_3 continuem aumentando (FAO, 2010; Bosland e Votava, 2012).

1.4.2. *Capsicum baccatum* L. var. *pendulum*

A espécie de pimenta *Capsicum baccatum* L. var. *pendulum* (Figura 2) compreende dois acessos (menor grupo taxonômico) bem diferentes conhecidos como: “pimenta-chapéu-de-frade” e “pimenta-dedo-de-moça”. A pimenta-chapéu-de-frade é uma pimenta com baixo teor de capsaicina e por isso é considerada uma pimenta doce, já a pimenta-dedo-de-moça tem quantidades variáveis de capsaicina variando de moderado a alto e por isso é considerada uma pimenta picante. A pimenta-dedo-de-moça é rica em compostos antioxidantes, como flavonóides, carotenóides e capsaicinóides (Wahyuni et al., 2011, 2013). Além disso, esta pimenta mais é uma das pimentas mais consumidas no Brasil, principalmente nas regiões Sul e Sudeste. Sua produção vem crescendo no Brasil, especialmente nos estados de Minas Gerais, Goiás, São Paulo, Ceará e Rio Grande do Sul (Carvalho et al, 2003).

Figura 2: frutos da espécie *Capsicum baccatum* L. var. *pendulum*.

1.5. Referencial Teórico

Esta seção é composta por um capítulo de livro, o qual serve como base teórica para a dissertação.

Título do livro: Abiotic Stress and their Management for Sustainable Agriculture

Capítulo: 8

Título do Capítulo: The impact of ozone pollution on plants defense metabolism: detrimental effects on yield and quality of agricultural crops

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CHAPTER 8

The impact of ozone pollution on plants defense metabolism: detrimental effects on yield and quality of agricultural crops

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Abstract

Over the past decades, research on the negative effects of air pollutants on agricultural crops and agro-ecosystems point out for emission reduction strategies, with practical recommendations to increase the sustainability of agricultural and land management in an environment that is constantly changing. Agricultural production will need to keep pace with the growing food demand, which depends on many factors, including the future levels of air pollution, such as tropospheric ozone. The risk of negative effects of ozone on crop productivity created the need to improve our understanding on the mechanisms underlying ozone toxicity, and biotechnological advances are now starting to provide us the necessary knowledge to safely develop and/or select crops varieties better adapted to ozone stress. Ozone phytotoxicity arises mainly because its high oxidation potential to generate reactive oxygen species (ROS) in exposed plant tissue. After enter leaf stomata, ozone rapidly degrades into various ROS species, and plants reduce the oxidative damage by activation of antioxidant enzymes and accumulation of molecules that effectively scavenge ROS. If ROS production exceeds the plant's capacity to detoxify it, deleterious effects at the cellular level may occur. The balance between the production and the scavenging of activated oxygen is thus crucial to plant growth maintenance and overall environmental stress tolerance. However, alterations in plant metabolism may lead to reduced crop yield and quality, directly or indirectly by exposing susceptible plants to stress factors. Secondary metabolites are constitutively synthesized and are of interest for human health and nutrition, especially because some of them are major source of biologically active substances. However, they are also well-known as plant defense molecules and their concentrations can be influenced by abiotic stresses such as ozone. Increased accumulation plant secondary metabolites in leaves of forest trees in response to ozone exposure has been reported in several studies, while the changes on crop plants composition and nutritional quality needs to be further studied and discussed to guide our efforts to select ozone-tolerant crops in an attempt to provide a secure food supply for a developing world.

1. Introduction

Tropospheric ozone is a major secondary air pollutant formed by the chemical reaction between nitrogen oxides (NOx) and volatile organic compounds (VOCs) in the presence of sunlight. Ground-level ozone concentrations have significantly increased since pre-industrial times, and in the northern hemisphere the mean ozone concentrations have raised from 10–15 ppb to current levels above 40 ppb (Ashmore, 2005; Vingarzan, 2004). According the modeling studies presented on the Intergovernmental Panel on Climate Change (IPCC, 2007), projections based upon scenarios with high emissions of primary pollutant species derivate from anthropogenic activity (NOx, CH_4, CO and VOCs) indicate that concentrations of tropospheric ozone might increase throughout the 21st century, and simulations for the period of 2015 through 2050 indicate an increase in ozone levels of 20 to 25%, whereas through 2100 the ozone levels below 250 mb (an altitude around 10 km) may grow by 40 to 60%. Therefore, ozone concentrations will probably exceed the internationally accepted environmental criteria (ranging around 40-50 ppb), which represents a significant risk for human health, natural vegetation and crops production (WHO, 2005).

On a global scale, pollution by ozone was considered largest in Central Europe and eastern United States of America, but recent trends in ozone concentration obtained trough global photochemical modeling studies performed for the Hemispheric Transport of Air Pollution 2010 assessment, indicated reductions in peak surface ozone levels in North America and Europe (Dentener *et al.*, 2010). These changes are likely to have been due to effective emission controls on primary air pollutants over the past two decades in response to the Clean Air Act in the United States and the Long-Range Transboundary Air Pollution Convention and European Union targets in Europe (Ashmore, 2005; Collins *et al.*, 2000; Vingarzan, 2004). However, in several developing countries we observe a different scenario, and emissions of ozone precursors are going upward as a consequence of rapid urbanization and industrialization across these regions (UNEP, 1999). The concentrations of air pollutants in some cities located in South Asia, India, and Latin America often exceed the thresholds of toxicity to human and ecosystems health (Agrawal *et al.*, 2003; Ashmore, 2005; Emberson *et al.*, 2001).

During the past decades the impacts of ozone have assumed great concern, and tropospheric ozone is now recognized as the most harmful air pollutant to crop plants and ecosystems. Despite control measures intended to reduce ozone pollution, current ground-level ozone concentrations in several countries worldwide leads to growth and yield impairment of many agricultural and horticultural plants, affecting crop productivity in regions where the agricultural production is the dominant economic activity (Booker *et al.*, 2009; Rai and Agrawal, 2012). Data collected from large-scale experimental studies conducted in filtration and fumigation chamber experimental studies performed by the North American Crop Loss Assessment Network (NCLAN) and the European Open Top Chamber Programme (EOTC) have estimated that the yields of about one third of U.S. crops were reduced by10% due to ambient ozone in the 1980s (EPA 1996), whereas the European Union (EU) may have lost more than 5% of

their wheat yield due to ozone exposure concentrations during the 1990s (Krupa *et al.*, 1998). Recently, Avnery and collegues (2011) estimated that the global yield reductions of three key staple crops due to surface ozone exposure using hourly ozone concentrations simulated by the Model for Ozone and Related Chemical Tracers version 2.4 (MOZART-2) and have found that detrimental impacts of ozone were already responsible for reductions of global yields for maize (ranging from 2.2–5.5%), wheat (3.9–15%) and soybean (8.5–14%) in 2000.

The increasing emissions of reactive VOCs and NOx in urban areas have significantly increased ozone concentrations in rural areas, and nowadays ozone levels are found to be higher in agricultural land than in cities (Ainsworth *et al.*, 2012). These are the case for many of regions located in the major crop-growing areas of Asia, India, Africa and Latin America. According to Emberson *et al.* (2009), ambient ozone concentrations in South Asia range between 35–75 ppb (4–8 h growing season mean), and the modelling-based studies performed by the authors suggest that yield losses of 5–20% of important crops are predicted to become common in Asia areas experiencing elevated ozone concentrations. Using HANK model for ozone concentration, Mittal *et al.* (2007) reported ozone levels varying from 25 to 100 ppb in the Indian sub-continent (Afghanistan, parts of Southeast Asian countries, and parts of China and Sri Lanka). The magnitude of potential risk of ozone to plant productivity and food safety in India was revised by Oksanen *et al.* (2013), and as showed by Sarkar and Agrawal (2012) current ozone concentrations severely affect growth, reproductive, physiological, molecular and yield parameters on two Indian rice cultivars. In Latin America, where crop and livestock production continues to expand, data concerning the effects of ozone on yield losses are still scarce. However, according to global distribution of crop exposure to ozone presented by Avnery *et al.* (2011) the highest exposure of crops to ozone generally occur in the Northern Hemisphere and Brazil due to greater ozone-precursor emissions and concentrations during the crops growing season. Ozone exposure during the soybean and maize growing seasons is high in the Northern Hemisphere, whereas in the Southern Hemisphere, the high ozone levels occurs during the periods of high biomass burning (August and October) which are coincident with maize growing season in Democratic Republic of the Congo and the wheat growing season in Brazil.

2. The basis of ozone detrimental effects

Since the early studies on the effects of ozone on plant species, it was observed that this pollutant is by nature a strong oxidizing agent capable of being rapidly converted in the intracellular space to different reactive oxygen species (ROS) (Castagna and Ranieri, 2009; Iriti and Faoro, 2008). Ozone movement into the apoplastic space is largely controlled by stomatal gas exchange, and immediately after its entry in the sub-stomatal chamber, it is spontaneously decomposed to ROS, such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$), and nitric oxide (NO), or it can react with a number of compounds present in cell wall,

apoplastic fluid and plasma membrane (Castagna and Ranieri, 2009; Laisk *et al.*, 1989; Sharma *et al.*, 2012). Studies performed with different species have reported that following ozone exposure (100 - 150 ppb) both H_2O_2 and $O_2^{\bullet -}$ are extensively accumulate in the leaf tissue, especially in sensitive plants (Caregnato *et al.*, 2013; Guidi *et al.*, 2010), and Scebba *et al.* (2003) reportes that extracellular ROS accumulation is one of the earliest detectable responses to ozone. Mahalingam *et al.* (2006) observed that ozone elicits a biphasic ROS burst in *Arabidopsis* with a smaller peak at 4 h and a larger peak at 16 h, and $O_2^{\bullet -}$ was the major ROS generated in response to 150 ppb of ozone. The direct harmful effects of ozone on leaves thus depend on the stomatal ozone flux, which is largely dependent on the gradient of ozone from outside to inside the leaf (Tuzet *et al.*, 2011). The reactions of ozone within the aqueous matrix of the cell wall (the apoplast) with extracellular antioxidants may control the actual amount of ozone that can reach the cell membrane, thereby changing the rate of ozone uptake via stomata (Tuzet *et al.*, 2011), and apoplastic ROS quenching antioxidant capacity can be considered the first line of defense against ozone harmful damages (Dizengremel *et al.*, 2008).

Following transient exposure to high levels of ozone, the overproduction of ROS can lead to oxidation of membrane lipids, proteins and enzymes, as well as a variety of organic metabolites localized into the cell. The initial signals produced by ozone can thus be later translated in responses at the tissue level, leading to hypersensitive response, accelerated senescence and programmed cell death (Mahalingam *et al.*, 2006). Despite their destructive activity, ROS are well-described as second messengers in a variety of cellular processes, which includes tolerance to abiotic and biotic environmental stresses (Guidi *et al.*, 2010; Wohlgemuth *et al.*, 2002). Besides the antioxidant enzymatic systems found in different cellular compartments, plants possess a range of antioxidant metabolites and detoxifying apparatus responsible for scavenging ROS. Plants can limit ozone-induced damages by protective mechanisms that involve the accumulation of compounds with high reducing potentials like ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and secondary metabolites, such as phenolic compounds (Baier *et al.*, 2005). Moreover, ozone response pathways overlaps with the programmed cell death induced in response to plant pathogens, and both stresses can induce the oxidative burst that leads to excessive ROS production, which activates the biosynthesis of ethylene, salicylic acid, and jasmonic acid (Kangasjarvi *et al.*, 2005; Sharma and Davis, 1997). These plant hormones coordinate different metabolic pathways involved in cell defense, and current evidences suggests that ethylene promotes endogenous ROS formation and lesion propagation, salicylic acid is required for programmed cell death, and jasmonic acid are involved in cell lesion containment (Baier *et al.*, 2005; Rao, 2000).

A number of authors have pointed out that the main level of ozone defense relies both on the existing content of cellular antioxidants (e.g. AsA and GSH) and the intensity of the detoxifying pathways that are responsible for regenerating these metabolites (Calatayud *et al.*, 2001; Dizengremel *et al.*, 2008, 2009; Luwe *et al.*, 1993).

The protective role of AsA as ROS-scavenger was first supported by the enhanced ozone-sensitivity shown by *Arabidopsis thaliana* mutants deficient in AsA synthesis (Conklin *et al.*, 1996). Even so, the relationship between ozone sensitivity and apoplastic AsA concentration remains controversial and some studies have postulated that elevated apoplastic AsA levels cannot always be sufficient to render a plant tolerant to ozone (D'Haese *et al.*, 2005; Di Baccio *et al.*, 2008; Ranieri *et al.*, 1999). The apoplast can be easily and rapidly depleted of AsA, allowing the subsequent oxidative action of ROS in foliar cells (Van Hove *et al.*, 2001), and thus an efficient protective mechanism requires the transfer of AsA from intracellular detoxifying systems to the cell wall. The antioxidant role played by AsA is known to be dependent on the cell ability to maintain it in a reduced state, which occurs trough the Halliwell-Asada cycle (AsA – GSH) (Di Baccio *et al.*, 2008; Noctor and Foyer, 1998; Smirnoff, 1996). Using high ozone concentrations (300 ppb) Luwe *et al.* (1993) observed a time-dependent relationship between oxidation of both extracellular AsA and intracellular GSH pool, while the cellular AsA redox state was unaltered during fumigation. As reported by numerous studies, AsA regeneration is tightly coupled to GSH within the cell and transport activity was responsible for replenish the reduced apoplastic AsA pool (for review see Noctor and Foyer, 1998; Smirnoff, 1996). In the symplasm, GSH and NAD(P)H are responsible for reducing the AsA molecule. The reduction of GSSG (oxidized GSH) into GSH occurs through the action of glutathione reductase (GR), and together with other enzymes like thioredoxins and peroxiredoxins, the GSH/GSSG couple plays a redox sensor role (Foyer and Noctor, 2005). In fact, regeneration of reduced AsA and GSH can be provided by enzymes that use the reducing power of NAD(P)H, which clearly appears as a key regulator in most regeneration processes (Noctor, 2006).

Thus, the capacity of cells to appropriately keep the antioxidant levels depends on carbon metabolism changes concomitant with alteration in gene expression (Foyer and Noctor, 2005). In higher plants, chronic ozone fumigation impairs the photosynthetic process and the carbon dioxide (CO_2) assimilation due to a decrease in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and quantity, together with the destruction of photosynthetic pigments (Anderson *et al.*, 2003; Calatayud *et al.*, 2007; Fontaine *et al.*, 1999; Iglesias *et al.*, 2006). While photosynthesis is limited, the activity and quantity of PEPcase (phosphoenolpyruvate carboxylase) is strongly increased, allowing accumulation of four-carbon acids (Dizengremel *et al.*, 2008). Several enzymes of glycolysis and the pentose phosphate pathway are also activated, providing precursors for the a anapleurotic pathway (oxaloacetate and malate) that will produce higher amounts of reducing power (NADPH and NADH) to further help the detoxification process (Dizengremel *et al.*, 2009). All together, ozone exposure lasting several days increase the levels of ROS, impairs the photosynthetic machinery and the Calvin cycle, causing the exhaustion of carbon availability as the demand for reducing power and energy are increased. In a meta-analysis study performed with data from 53 peer-reviewed works published between 1980 and 2007 that evaluated the responses of wheat (*Triticum aestivum*) to elevated ozone, Feng *et al.* (2008)

demonstrated that ozone exposure to an average concentration of 43 ppm significantly decreased photosynthetic rates (20%), Rubisco activity (19%), stomatal conductance (22%), and chlorophyll content (40%), and such biochemical modifications affected the whole plant by inducing a larger decrease in belowground (27%) biomass than in aboveground (18%) biomass.

Ozone-induced reductions in photosynthesis not only change carbon assimilation, but also affect carbon translocation and accumulation in different plant parts. This arise either from a reduction in carbon translocation from source leaves to distant sink, which, according to Grantz and Farrar (2000), occurs due to phloem inhibition transport; or from the effect of ozone on ethylene synthesis, a hormone that controls shoot and root growth, promote senescence and abscission, and more recently, has been associated with the disruption of ABA-induced stomatal opening regulation (Wilkinson *et al.*, 2012). The negative impacts on root biomass might lead to reductions in grain and fruit production, since the ability of the plant to take up the nutrients and water required to sustain growth and yield is compromised (Ashmore, 2005; Rai and Agrawal, 2012). Besides, under ozone stress the pool of non-structural carbohydrates essential for growth, including sugars and starch, are affected both due to reduction in the carbohydrate synthesis and by a shift of carbon compounds to repair processes and defense metabolites (Booker *et al.*, 2009; Fuhrer and Booker, 2003; Wang and Frei, 2011). The synthesis of defense metabolites might divert resources away from the synthesis of other sets of metabolites, so analysis of the plant metabolite profiling could be assessed to identify the tradeoffs between primary and secondary metabolism (Stitt *et al.*, 2010).

3. Ozone and the changes on plant defense metabolism

Probably one of the most important adjustments made by plants to avoid environmental stress is to change the chemical composition of leaves, flowers, fruits, roots and stems. In certain varieties of wheat, rice, bean, soybean and sorghum, the physiological stress imposed by ozone modifies the chemical composition of crops, affecting not only the grain size and weight, but also the nutritional composition of the final agricultural products (Betzelberger *et al.*, 2012; Biswas *et al.*, 2008; Booker *et al.*, 2009; Iriti *et al.*, 2009; Wang *et al.*, 2012). Ozone exposure can activate the biosynthesis of plant secondary metabolites, a diverse group of organic compounds with important adaptive significance in protecting plants against predators and pathogens, in providing reproductive advantage as attractants of pollinators and seed-dispersing animals, and as allelopathic agents (Croteau *et al.*, 2000; Harborne, 1993). Besides the importance for the plant itself, secondary metabolites determine a number of nutritional aspects of food, including color, taste, smell, and antioxidative, anticarcinogenic, anti-inflammatory and cholesterol-lowering properties (Hounsome *et al.*, 2008). Thus, shifts in the chemical composition of important field crops can lead to loss of potentially beneficial components and have detrimental impacts on food safety and consumer's health.

Based on their biosynthetic origins, plant secondary compounds can be divided into three major groups: the phenylpropanoids, the terpenoids and the alkaloids. Phytochemicals arising from these pathways include compounds with a powerful antioxidant capacity, able to efficiently scavenge different ROS (Di Baccio *et al.*, 2008; Iriti and Faoro, 2008; Prior *et al.*, 1998). Many phenolic compounds, which are primarily derived from phenylpropanoid pathway, are known to work as effective antioxidants molecules because the electron reduction potential of the phenolic radical is lower than the electron reduction potential of oxygen radicals, and also because phenoxyl radicals are generally less reactive than oxygen radicals (Rice-Evans *et al.*, 1997). Phenolic compounds such as flavonoids are responsible for determine distinguishing traits of plant parts, establishing, for example, flower colors, and leaves and grains flavors (tastes and odors).

In plants, the phenylpropanoid metabolism is induced in response to stress, and enhancement of key enzyme activities and accumulation of secondary metabolites occur early after exposure, in order to improve the resistance against pathogen attack and/or tolerance to environmental pollutants (Iriti and Faoro, 2009). Ozone can elevate the level of flux through the phenylpropanoid pathway stimulating the production of phenolic compounds, including lignin, suberin, tannin, stilbenes and flavonoids (Eckey-kaltenbach *et al.*, 1994; Saleem *et al.*, 2001; Tuomainen *et al.*, 1996). According to some studies, the phenylpropanoid pathway is one of the most affected targets of ozone, inducing genes transcription and enzymes activities (Di Baccio *et al.*, 2008; Tosti *et al.*, 2006). The shikimate dehydrogenase (SKDH) is one of the key enzymes of the shikimate pathway, a metabolic route that produces aromatic amino acids and a large number of phenolic compounds. Increased accumulation of flavonoids, such as quercetin and chlorogenic acid, has been found in different natural and cultivated plant species exposed to elevate ozone levels (Saleem *et al.*, 2001; Saviranta *et al.*, 2010), and as suggested by Appel (1993), this compounds further increase resistance against ozone damage by scavenging $OH^{\bullet}$ and H_2O_2. Increased levels of transcription of genes involved in flavonoid biosynthesis was also found in ozone resistant leguminous cultivars (Puckette *et al.*, 2008), suggesting that a number of transcription factors and signaling genes differently enable resistant plants to adapt more rapidly to ozone stress. Furthermore, Booker and Miller (1998) observed in a greenhouse study with soybeans that after 6h of ozone fumigation a rapidly and coordinated increase in the activities of two phenylpropanoid pathway enzymes, phenylalanine ammonia lyase (PAL) and 4-coumarate:CoA ligase, and that the stimulation of these enzymes activities remained elevated for several days.

Terpenoids are the most structurally diversified class of plant natural products, with functional roles in plants as structural components of membranes (sterols), electron carriers (quinones), photosynthetic pigments (phytol, carotenoids), and hormones (gibberelins, abscisic acid) (Croteau *et al.*, 2000). Isoprenoids have several bioactive properties and thus have long been used in pharmacological industry and in the human diet (Hounsome *et al.*, 2008). In Plants, isoprene may act as protecting thermal agents,

but a more general antioxidant action has been recently hypothesized on the basis of protection against abiotic stress (Spinelli *et al.*, 2011). However, the response of isoprenoid biosynthetic pathway to ozone may vary considerably, and according to Calfapietra *et al.* (2009) it is especially dependent on the length and level of exposure to the pollutant. Measurements of isoprene emission carried out in *Populus tremuloides* chronically exposed to ozone (1.5- fold the ambient levels for several years) indicated that isoprene synthesis and emission were decreased, and such response were associated with reductions in isoprene synthase messenger RNA and reduced levels of dimethylallyl diphosphate (DMADP), the main substrate for isoprene synthesis (Calfapietra *et al.*, 2008, 2007). Puckett *et al.* (2008) reported that in *Medicago truncatula* ozone-resistant accession exposed to acute ozone treatment (300 nL L^{-1} for six hours), key genes related to isoprenoid biosynthesis pathway were strongly up regulated at 12 hours pos-treatment.

Alkaloids are nitrogen-containing compounds mainly derived from amino acids, which possess great interest because of their pronounced toxicological, pharmacological, nutritional and medicinal properties (e.g. caffeine, nicotine, morphine, quinine). Most alkaloids are very toxic and, therefore, they are found to play important role in plant chemical defense against herbivores and microorganisms (Harborne, 1993). Glycoalkaloids such as α-solanine and α-chaconine, for example, are naturally occurring phytotoxins in potato that may cause a bitter taste and gastroenteritis. For food safety purposes an upper limit for total glycoalkaloid content of 20 mg per 100 g of potato is generally accepted, if they occur in too high concentrations can be considered lethal to humans (Friedman and McDonald, 1997; Sinden *et al.*, 1976; Smith *et al.*, 1996). Studies concerning the effects of ozone on the alkaloids biosynthetic pathway are still scarce, and most of them deal with the influence of the pollutant on the nitrogen metabolism (see Iriti and Faoro, 2009). In a study with tobacco plants (*Nicotiana tabacum*) grown under high ozone concentrations (80-100ppb) the authors observed that treated plants had higher levels of total nitrogen (primarily reduced nitrogen) and lower levels of nicotine (a pyridine alkaloid), which increased the survival and growth response of tobacco hornworm larvae (*Manduca sexta*) once the plant chemical defense contents were modified by the pollutant (Jackson *et al.*, 2000). In addition, Langebartels *et al.*(1991) observed that a single ozone treatment (5 or 7 hours) had a strong influence on the levels of polyamines (putrescine and spermidine), important alkaloid precursors, that can improve ozone tolerance either acting as ROS scavengers molecules or inhibiting the ethylene biosynthesis and reducing thus the senescence.

As secondary metabolites are products of primary metabolism, an excessive activation of defense compounds biosynthesis can have detrimental effects on plant fitness-relevant functions such as growth and reproduction (Bolton, 2009). If priority is given to the defense-related processes the availability of carbon and nitrogen resources may become limiting (Le Bot *et al.*, 2009; Manderscheid *et al.*, 1992). As observed by Saleem *et al.* (2001), long-term ozone exposure of sensitive silver birch clone (*Betula pendula* Roth) increase total phenolic content (16.2%) at the expense of growth,

suggesting that changes in carbon allocation towards chemical defense resulted in lower biomass production. Under chronic ozone exposure, shifts in the partitioning of photosynthates may severely influences the content of carbohydrates and minerals on roots and lower leaves mainly because carbon compounds allocation to young leaves and seed production are necessary to maximize resource acquisition to survival. Such changes are known to be dependent on environmental factors such as temperature, soil nutrients, nitrogen availability and water stress (for review see Bender and Weigel, 2011; Fuhrer and Booker, 2003; Ainsworth *et al.*, 2012).

4. The metabolic shift and the influences on plant quality

The negative impacts of ozone on yield have become a great threat to global food security, especially in developing countries, where food shortages are a risk in the face of rapidly increasing populations. However, elevate ozone levels not only threatens agriculture and food security by reducing the food quantity, but also by changing the food quality. While the ozone negative impacts on crop yields are obvious, their effects on crop quality are almost unknown (Ashmore, 2005).

Crop quality may be affected either by changes in primary metabolite production (e.g. carbohydrates, proteins), or as a consequence of increased secondary metabolism synthesis. Ozone-induced deviations of available resources from growth to defense metabolism might alter the chemical composition of crops and consequently the quality of harvested products of crops (Iriti and Faoro, 2009; Wang and Frei, 2011). Phytochemicals arising from defense (secondary) metabolism are important for human health and nutrition, especially because some of them are source of biologically active substances not only with health-benefits potential, such as chemopreventive, anti-inflammatory and antioxidants, but also health-harmful potential, like carcinogenic and toxic compounds (Chen and Kong, 2004; Crozier *et al.*, 2006; Korkina, 2007).

Among the few studies that assess the quality of the marketable crop products (grains, tubers, fruits and vegetables) most of them investigates the content of proteins, carbohydrates and lipids, while very few evaluate the change on secondary compounds content. Visible injuries induced by ozone are also of great importance especially when the marketable value of the crop depends on the appearance. For example, in leafy vegetables visible injuries may make the product unmarketable (Ashmore, 2005). In addition, the ozone may alter the quality of forages making them less digestible and less nutritious for ruminants, allowing the emergence of ozone secondary effects, such as reduction in the milk and meat production from grazing animals (Vandermeiren and Pleije, 2011). Here we divided the ozone impacts on crop quality into seven categories of quality parameters, which according Wang and Frei (2011) are: protein, carbohydrates, lipids, minerals, secondary compounds, physical and sensory aspects, and nutritive value of forage for ruminant animals.

4.1. Proteins

Plant foodstuffs are a great source of dietary protein for humans and animals. On a global basis, plant proteins provide about 60% of the human daily protein intake, mainly from cereal grains. However, in developing countries this value may be even higher (FAO, 2009). Therefore, protein content plays a significant role in determining the nutritional quality of many crops, especially in developing countries.

Plants exposed to ozone are known to have protein concentration of harvested fractions altered. Generally, an increment in the amount of grain proteins is often associated with crops exposed to ozone, as seen in wheat, soybean, bean and rice (Table 1). However, this increase is not large enough to compensate the grain yield loss, so the grain protein yield is reduced (Feng *et al.*, 2008; Frei *et al.*, 2012; Mulchi *et al.*, 1988; Piikki *et al.*, 2008; Pleijel *et al.*, 1997; Zheng *et al.*, 2013). Differently from the grains, the protein concentration in leaves does not show a trend as seen in the Table 1.

In wheat (*Triticum aestivum*), which is considered to be one of the most ozone-sensitive crops (Mills *et al.*, 2007), changes on grain protein content are a very important effect elicited by ozone, especially because it is a major source of plant protein worldwide (FAO, 2009; Vandermeiren and Pleije, 2011). The protein concentration usually increases in wheat grain of plants grown under ozone exposure, while in leaf it is reduced (Table 1). Zheng *et al.* (2013) discuss that the higher protein levels in grains are likely a consequence of reduced carbohydrate levels. In addition, there are indications that not only the amount, but also the composition of the proteins is affected by ozone, for example, the dry gluten/protein ratio was increased in wheat grains from plants grown at ambient ozone levels (Vandermeiren *et al.*, 1992). Moreover, Fuhrer *et al.* (1992) found a small but significant increase in Zeleny values with increasing ozone concentration indicating a trend towards better protein quality.

Rice is listed as the grain crop with the second highest world production (FAO 2009) providing over 21% of the caloric needs of the world's population and up to 76% of the caloric intake of South East Asia population (Fitzgerald *et al.*, 2009). Mills *et al.* (2007) identified rice as a moderately sensitive staple crop, and a number of studies with different cultivars found that, not only yield, but other major growth parameters are severely affected by ozone (Rai *et al.*, 2010; Sarkar and Agrawal, 2012). Rai *et al.* (2010) observed a reduction in seed protein concentration in two rice cultivars grown in non filtered chambers (NFC) when compared to filtered chambers (FC). However, these results contrast with those reported by Zheng *et al.* (2013), Frei *et al.* (2012) and Wang *et al.*(2012), who showed increments in seed protein levels of rice plants grown in NFCs.

Table 1: Effect of ozone stress on the protein content of major crops and forage.

	Crop species	Organ	Ozone effect	References
Protein	Bahiagrass, *Paspalum notatum* Flugge	Leaf	– / ↑	Muntifering *et al*. (2000)
	Bean, *Phaseolus vulgaris* L.	Seed	↑	Iriti *et al*. (2009)
	Broccoli, *Brassica oleracea* L.	Flower + stalk	↑	Vandermeiren *et al*. (2012)
	Clover, *Trifolium subterraneum* L.	Leaf	↑	Sanz *et al*. (2005)
	Corn, *Zea mays* L.	Seed	–	Garcia *et al*. (1983)
	Grass, *Briza maxima*	Leaf	↓	Sanz *et al*. (2011)
	Grassland species mixture	Leaf	↓	Gilliland *et al*. (2012)
	Lespedeza, *Lespedeza cuneata*	Leaf	–	Powell *et al*. (2003)
	Little bluestem, *Schizachyrium scoparium*	Leaf	↓	Powell *et al*. (2003)
	Mustard, *Brassica campestris* L.	Seed	↓	Singh *et al*. (2009), Tripathi and Agrawal (2012)
	Peanut, *Arachis hypogeal* L.	Seed	–	Burkey *et al*. (2007)
	Rapeseed, *Brassica napus* L.	Seed	– / ↓ / ↑	Bosac *et al*. (1998), Ollerenshaw *et al*. (1999), Vandermeiren *et al*. (2012)
	Rice straw, *Oryza sativa* L.	Leaf	↑	Frei *et al*. (2011)
	Rice, *Oryza sativa* L.	Seed	↓ / ↑	Rai *et al*. (2010), Frei *et al*. (2012), Wang *et al*. (2012), Zheng *et al*. (2013)
	Soybean, *Glycine max* (L.) Merr.	Seed	– / ↑	Howell and Rose (1980), Grunwald and Endress (1984), Mulchi *et al*. (1988)
	Wheat, *Triticum aestivum* L.	Seed	– / ↑	Fuhrer *et al*. (1990,1992), Pleijel *et al*. (1991, 1997, 1998, 1999, 2006), Feng *et al*. (2008), Piikki *et al*. (2008), Zheng *et al*. (2013)
	Wheat, *Triticum aestivum* L.	Leaf	↓	Feng *et al*. (2008)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on protein content.

Besides wheat and rice, legumes are great sources of protein being up to three times richer in protein than cereals grains (Duranti and Gius, 1997). Analyzing the Table 1 it is possible to note that protein concentration in legumes grains increase when plants are exposed to ozone, which is the case for soybean and bean. However, seeds from peanut (*Arachis hypogaea* L.), which is considered to be sensitive to ozone, do not have their protein content modified by the pollutant (Burkey *et al.*, 2007). In contrast, the ozone tends to decrease protein content in seeds of *Brassica* genus as shown by Bosac *et al.* (1998), Ollerenshaw *et al.* (1999), Singh *et al.* (2009), and Tripathi and Agrawal (2012), although Vandermeiren *et al.* (2012) have shown that ozone increase protein content in rapeseed.

4.2. Lipids

With some exceptions, in contrast to animal fats, vegetable oils contain predominantly unsaturated fatty acids which are very important to human health. Some unsaturated fatty acids like linoleic acid (omega-6 family) and α-linolenic acid (omega-3 family) are essential for humans because we are not able to completely synthesize them. On the opposite, plants have this ability and plants products are the major source of essential fatty acids in human food chain. Thus, changes induced by ozone on plants lipid content should be considered.

As seen in Table 2, ozone effects on seed lipid content does not show a clear trend, although in studies with mustard and rapeseed, which are two major world sources of vegetable oils, ozone decreases lipid content in most cases (Bosac *et al.*, 1998; Ollerenshaw *et al.*, 1999; Singh *et al.*, 2009; Tripathi and Agrawal, 2012). Rapeseed oil is a valuable plant oil for human nutrition due to its high content of monounsaturated and polyunsaturated fatty acids combined with a very low proportion of saturated fatty acids (Vandermeiren *et al.*, 2012). A study conducted by Vandermeiren *et al.* (2012) showed that ozone led to a shift in fatty acid composition of the vegetable oil derived from seeds of oilseed rape. The authors observed that the content of oleic acid (18:1) significantly declined, linoleic acid (18:2) increased, and linolenic acid (18:3) had no differences. Total monounsaturated fatty acids were decreased by ozone exposure, while total saturated fatty acids were increased, leading oil quality decreases.

Table 2: Effect of ozone stress on the lipid content of major crops.

	Crop species	Organ	Ozone effect	References
Lipid	Bean, *Phaseolus vulgaris* L.	Seed	↑	Iriti *et al*. (2009)
	Corn, *Zea mays* L.	Seed	–	Garcia *et al*. (1983)
	Mustard, *Brassica campestris* L.	Seed	↓	Singh *et al*. (2009), Tripathi and Agrawal (2012)
	Peanut, *Arachis hypogeal* L.	Seed	–	Burkey *et al*. (2007)
	Rapeseed, *Brassica napus* L.	Seed	– / ↓ / ↑	Bosac *et al*. (1998), Ollerenshaw *et al*. (1999), Vandermeiren *et al*. (2012)
	Rice, *Oryza sativa* L.	Seed	↑	Frei (2012)
	Soybean, *Glycine max* (L.)	Seed	– / ↓	Howell and Rose (1980), Mulchi *et al*. (1988), Grunwald and Endress (1984)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on lipid content.

Singh *et al.* (2009) observed that in response to ambient ozone the contents of oil, protein and minerals (Ca, Mg, K, P, Zn) were significantly decreased in mustard seeds when compared to the plants grown in air filtered chambers at the recommended NPK (nitrogen, phosphorus and potassium) fertilization. However, these effects were suppressed when 1.5x of recommended NPK was added to the soil. Tripathi and Agrawal (2012) reported that in mustard seeds the fatty acid profile was altered by ozone, reporting that saturated fatty acid content was reduced after ozone exposure. However, monounsaturated fatty acid, polyunsaturated fatty acid and ω-6 fatty acid showed a gain after the treatment. Among the fatty acids components, linoleic acid was decreased whereas oleic, erucic and linolenic acid were enhanced in response to ozone. Lower levels of linolenic acid and higher contents of oleic acid is preferred for cooking and frying purpose (Nesi *et al.*, 2008). Some environmental factors like ozone are able to alter the seed oi:protein ratio. In soybean plant grown in ozone ambient the authors Howell and Rose (1980), and Grunwald and Endress (1984) found a significant lower oil:protein ratio in the seeds, which was associated with a decrease in seeds total oil content.

4.3. Carbohydrates

To analyze the ozone impacts on the carbohydrates quality in crop products we may separate them into three components: sugar, starch and fiber content. Ozone effects on fiber content of plant foodstuff for human consumption is the least studied, although their intake has important implications for health. For example, human consumption of soluble and insoluble dietary fibers has been related with weight loss, and some studies have found that a diet with higher insoluble fiber content can reduce the risk of bowel cancer and heart diseases (Ceyhan *et al.*, 2012).

Regarding the impacts on carbohydrates constituents, we observed that the majority of studies report that starch and reducing sugar (glucose and fructose) concentration decrease while fiber content is enhanced in many species exposed to ozone, despite experimental differences in ozone treatments (Table 3). Sucrose content showed no change except for the study conducted by Köllner and Krause (2000) who found that sucrose levels was decreased after a ozone exposure. Potato (*Solanum tuberosum*L.) has a great importance for human nutrition and, in terms of the production, is the fourth most important crop in global scale, coming after wheat, rice and maize (FAO, 2009). Potato tubers have several applications in the food industry, for which quality has a major importance. In this context, starch and reducing sugar of tuber play an important role to determine the potato tuber quality. The starch content of potato tubers must be sufficiently high to avoid excessive absorption of fat during frying, whereas the reducing sugar content should be low to prevent the darkening of chips due to the Maillard reaction, which are unacceptable in fried potato products (Roe *et al.*, 1990; Vandermeiren *et al.*, 2005).

Table 3: Effect of ozone stress on the carbohydrates content of major crops, forages and wooden trees.

Carbohydrates	Crop species	Organ	Ozone effect	References
Starch	Corn, *Zea mays* L.	Seed	–	Garcia *et al*. (1983)
	Potato, *Solanum tuberosum* L.	Tuber	↓ / –	Pell and Pearson (1984), Köllner and Krause (2000), Vorne *et al*. (2002), Vandermeiren *et al*. (2005)
	Rice, *Oryza sativa* L.	Seed	↓	Rai *et al*. (2010), Frei *et al*. (2012)
	Sweet Potato, *Ipomoea batatas* (L.) Lam.	Tuber	↓	Keutgen *et al*. (2008)
	Wheat, *Triticum aestivum* L.	Seed	↓	Fuhrer *et al*. (1990, 1992), Feng *et al*. (2008)
Reducing sugar (fructose and glucose)	Grape, *Vitis vinifera* L.	Fruit	– /↓	Soja *et al*. (1997), Soja *et al*. (2004)
	Ladino clover, *Trifolium repens* L.	"Shoot"	↓	Blum *et al*. (1982)
	Mustard, *Brassica campestris* L.	Seed	↓	Tripathi and Agrawal (2012)
	Potato, *Solanum tuberosum* L.	Tuber	↓ / ↑	Pell *et al*. (1980, 1988), Pell and Pearson (1984), Vorne *et al*. (2002)
	Rapeseed, *Brassica napus* L.	Seed	– / ↓	Bosac *et al*. (1998)
	Rice, *Oryza sativa* L.	Seed	↑	Rai *et al*. (2010)
	Sweet Potato, *Ipomoea batatas* (L.) Lam.	Tuber	– / ↓	Keutgen *et al*. (2008)
Sucrose	Potato, *Solanum tuberosum* L.	Tuber	– / ↓	Pell *et al*. (1980, 1988), Köllner and Krause (2000), Vorne *et al*. (2002), Vandermeiren *et al*. (2005)
	Strawberry, *Fragaria×ananassa* Duch.	Fruit	–	Keutgen and Pawelzik (2008)
	Sweet Potato, *Ipomoea batatas* (L.) Lam.	Tuber	–	Keutgen *et al*. (2008)
Fibre (NDF, ADF and lignin)	Bahiagrass, *Paspalum notatum* Flugge	Leaf	– / ↑	Muntifering *et al*. (2000)
	Bean, *Phaseolus vulgaris* L.	Seed	↑	Iriti *et al*. (2009)
	Clover, *Trifolium* spp.	Leaf	– / ↑	Sanz *et al*. (2005), Muntifering *et al*. (2006), Gonzalez-Fernandez *et al*. (2008)
	Corn, *Zea mays* L.	Seed	–	Garcia *et al*. (1983)
	Grass, *Briza maxima*	Leaf	↑	Sanz *et al*. (2011)
	Grassland species mixture	Leaf	– / ↑	Gilliland *et al*. (2012)
	Poa pratensis L.	Leaf	– / ↑	Bender *et al*. (2006)
	Lespedeza, *Lespedeza cuneata*	Leaf	↑	Powell *et al*. (2003)
	Little bluestem, *Schizachyrium scoparium*	Leaf	– / ↑	Powell *et al*. (2003)
	Rice straw, *Oryza sativa* L.	Leaf	↑	Frei *et al*. (2011)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on carbohydrates content.

Sucrose content may also contribute with Maillard reaction through their byproducts formed after sucrose hydrolysis induced by heat during frying (Leszkowiat *et al.*, 1990). According to Mills *et al.* (2007) potato is a moderately sensitive crop to ozone; even so, the pollutant is able to change tuber quality. Pell *et al.* (1980) and Pell and Pearson (1984) reported that ozone increases the reducing sugar content, while Pell *et al.* (1988) and Vorne *et al.* (2002) reported that ozone improves potato tuber quality by decreasing the content of reducing sugars. On the other hand, the reduction of the starch content observed in many studies might have a negative impact on tuber quality (Table 2). Similarly to potato, sweet potato starch and reducing sugar content decreased while sucrose did not change after ozone exposure (Table 2). Ozone decreased reducing sugar content in grains of both *Brassica* species, unlike rice grain where reducing sugar content was enhanced (Table 2). Fuhrer *et al.* (1992, 1990) and Feng *et al.* (2008) reported lower starch content in wheat grains in response to ozone.

Quality is a major determinant of fruit crop value (Soja *et al.*, 2004), and in strawberry, for example, fruit carbohydrate and sugar compounds profile play an important role on flavor and quality (Keutgen and Pawelzik, 2007). Concentrations of sucrose and glucose in two different cultivars of strawberry (cv. Korona and cv. Elsanta) were not significantly influenced by ozone, while fructose content decreased in fruit of cv. Elsanta grown under ozone exposure. Although there were no changes in sucrose and glucose content, the authors found that ozone pollution during growth phase tended to reduce the sweetness index in both cultivars (Keutgen and Pawelzik, 2008). In grape berries, the accumulation of reducing sugar showed a greater decrease than grape yield at the highest ozone exposure in most experimental replicates (Soja *et al.*, 2004, 1997).

4.4. Secondary compounds

In the last years, a number of studies have pointed out the benefits of phytochemicals to human health, which are considered to have the ability to acts as anti-inflammatory, antioxidant, antiviral and anticancer agents. However, some secondary compounds, like alkaloids, might be very toxic for us, and many of the functions of secondary metabolites remain unknown and still need to be elucidated (Crozier at al., 2006; Hounsome *et al.*, 2008). Thus, ozone-mediated changes in the composition of plants secondary compounds may represent a risk for human consumption.

Changes in the composition of the secondary compounds elicited by ozone exposure are presented on the Table 4. Phenolics have been the most extensively studied metabolites. These molecules are derived from the phenylpropanoid biosynthetic pathway, which is strongly responsive to diverse environmental stress (Korkina, 2007).

Table 4: Effect of ozone stress on the secondary compounds and vitamins of major crops, forages and wooden trees.

Secondary compounds and vitamins	Crop species	Organ	Ozone effect	References
Alkaloids	Potato, *Solanum tuberosum* L.	Tuber	– / ↑ / ↓	Speroni *et al*. (1981), Pell and Pearson (1984), Donnelly *et al*. (2001), Vorne *et al*. (2002), Vandermeiren *et al*. (2005)
Ascorbate (vitamin C)	Broccoli, *Brassica oleracea* L.	Flower + stalk	–	Vandermeiren *et al*. (2012)
	Lettuce, *Lactuca sativa* L.	Leaf	↓	Calatayud *et al*. (2002)
	Potato, *Solanum tuberosum* L.	Tuber	– / ↑	Vorne *et al*. (2002)
	Spinach, *Spinacia oleracea* L.	Leaf	↓	Calatayud *et al*. (2003)
	Strawberry, *Fragaria×ananassa* Duch.	Fruit	↓	Keutgen and Pawelzik (2008)
Carotenoids	Sweet Potato, *Ipomoea batatas* (L.) Lam.	Leaf	↓ / –	Keutgen *et al*. (2008)
Glucosinolate	Broccoli, *Brassica oleracea* L.	Flower + stalk	– / ↑ / ↓	Vandermeiren *et al*. (2012)
	Rapeseed, *Brassica napus* L.	Seed	–	Vandermeiren *et al*. (2012)
	Rapeseed, *Brassica napus* L.	Leaf	– / ↑ / ↓	Gielen *et al*. (2006), Himanen *et al*. (2008)
Phenolic	Bahiagrass, *Paspalum notatum* Flugge	Leaf	–	Muntifering *et al*. (2000)
	Bean, *Phaseolus vulgaris* L.	Seed	↑	Iriti *et al*. (2009)
	Clover, *Trifolium* spp.	Leaf	– / ↑	Muntifering *et al*. (2006), Saviranta *et al*. (2010)
	Lespedeza, *Lespedeza cuneata*	Leaf	–	Powell *et al*. (2003)
	Little bluestem, *Schizachyrium scoparium*	Leaf	– / ↓	Powell *et al*. (2003)
	Rice straw, *Oryza sativa* L.	Leaf	↑	Frei *et al*. (2011)
	Silver Birch, *Betula pendula*	Leaf	↑	Saleem *et al*. (2001)
	Soybean *Glycine max* L.	Leaf	↑	Keen and Taylor (1975), Booker and Miller (1998)
	Strawberry, *Fragaria×ananassa* Duch.	Fruit	–	Keutgen and Pawelzik (2008)
	Rice, *Oryza sativa* L.	Seed	–	Frei *et al*. (2012)
	Tobacco, *Nicotiana tabacum* L.	Leaf	↑	Langebartels *et al*. (1991)
Tocopherol (vitamin E)	Broccoli, *Brassica oleracea* L.	Flower + stalk	–	Vandermeiren *et al*.(2012)
	Wheat, *Triticum aestivum* L.	Seed	–	Fuhrer *et al*. (1990)
	Rapeseed, *Brassica napus* L.	Seed	↓	Vandermeiren *et al*. (2012)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on secondary compounds and vitamins content.

The majority of studies tabulated herein reported an increase or no changes on phenolics in the edible fractions of a variety of crops grown under ozone exposure, except in leaves of little bluestem (primary-growth) reported by Powell *et al.* (2003). In most cases, PAL is the main enzyme responsible by the accumulation of phenolics in agricultural products produced under stressful conditions. Booker and Miller (1998) observed in soybean leaves an increase in PAL activity after 6 h of ozone treatment, and these activities remained elevated for several days. In the same study they found a continuous increase throughout ozone exposure showing a relationship between phenolic content and PAL activity.

The majority of studies were performed in leaves while only three studies assessed other parts of plant, like fruit and seed. Among these three works, Iriti *et al.* (2009) conducted the most complete work regarding secondary compounds. They evaluated the ozone effect in bean seeds, and have found an increase in total phenolic content and changes in phenolics proportion. Separately, the majority of phenolics assessed (delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, kaempferol, kaempferol-3-glucoside, caffeic acid, p-coumaric acid and sinapic acid) decreased while only two of them (petunidin-3-glucoside and pelargonidin-3-glucoside) increased. The authors also observed an increase in antioxidant potential; however, they suggested that this change was unrelated to the modification in the phenolic compounds. In tobacco leaves three phenolic compounds showed to be elevated when plants grown under ozone exposure. Caffeoyl-putrescine, which represents the major phenolic component of the apoplastic fluid of leaves, was four-fold increased after ozone treatment (Langebartels *et al.*, 1991).

Apoplastic ascorbate content, which is thought to be the first line of defense against ROS in leaves (Castagna and Ranieri, 2009), declined 15 % in spinach leaves and 35 % in lettuce leaves grown under ozone fumigation (Calatayud *et al.*, 2003, 2002). In potatoes, the amounts of ascorbic acid are moderate, but because of the high consumption in some regions in Europe it is one of most important sources of vitamin C (FAO, 2009; Lee and Kader, 2000). Increases in ascorbate content in tuber of potatoes plant exposed to ozone observed by Vorne *et al.* (2002) indicate an improvement in the tuber quality. In strawberry fruit, Keutgen and Pawelzik (2008) observed that the levels of total ascorbic acid significantly decreased in ozone exposed plants of two different cultivars (cv. Korona and cv. Elsanta). Tocopherols (vitamin E) are powerful antioxidants, therefore a decrease in vitamin E could be considered as a negative effect on plants nutritional value. Vitamin E in oilseed rape was significantly reduced at increasing ozone concentrations (Vandermeiren *et al.*, 2012). In contrast, broccoli and wheat did not show any difference in the vitamin E content in plants grown under high levels of ozone.

Glycoalkaloids are phytoxins naturally found in potato, and in most cases, the total glycoalkaloids concentrations in tuber is not significantly affected by ozone (Donnelly *et al.*, 2001; Speroni *et al.*, 1981; Vorne *et al.*, 2002). Although Donnelly *et*

al. (2001) did not find difference in total glycoalkaloids, the authors observed an increase in α-solanine content and no difference in α-chaconine content. Pell and Pearson (1984) observed that the response to the ozone depends on the cultivar, and total glycoalkaloid content decreased in tubers of cv. Norchip and increased in those of cv. Cherokee.

Glucosinolates, a group of nitrogen- and sulphur-containing secondary compounds involved in chemical protection against herbivores and stress, are characteristic to the Brassicaceae and some families of the Capparales order. These metabolites are toxic to some animals (including humans) in high concentration, but in low concentrations seems to have benefits to humans health such as anti-cancer properties (Sarıkamış, 2009; Tripathi and Mishra, 2007). Vandermeiren *et al.* (2012) observed that the total glucosinolate content of the rapeseed seeds and broccoli head was not significantly changed when plants were grown under ozone exposure, although in broccoli head, the ozone exposure increased the aliphatic glucosinolate and decreased the indolic glucosinolate. In leaves of rapeseed plants chronically exposed to ozone a clear change in the glucosinolate profile was found, although no changes in total glucosinolate could be observed (Himanen *et al.*, 2008). Gielen *et al.* (2006) in a study with two lines of *Brassica napus* L. subspecies *oleifera* with different concentrations of glucosinolates, and observed that ozone exposure only changed the glucosinolate content in the line with high glucosinolate concentration, which diminished the leaf total glucosinolate content in the presence of ozone.

4.5. Minerals

Human adequate mineral intake is needed for good health and to prevent nutritional disorders. Agricultural products are rich sources of several essential minerals, hence a large number of studies have measured mineral concentration of the edible parts of different crops. It is known that mineral content are influenced very much by surrounding environment, however, few studies have investigated the interactions between mineral concentration of the edible parts of crop and ozone stress (Ceyhan *et al.*, 2012; Wang and Frei, 2011).

Although the effect of ozone on mineral is important, we do not find any pattern of influence, making it difficult to properly discuss the results found so far, and more studies are needed to fill the gaps and provide robustness to data. Even so, the results concerning the mineral content of plants exposed to ozone are presented in the Table 5. Regarding minerals content, seeds of wheat and rice were the most studied agricultural products. In wheat grains ozone increase 7 of 9 studied minerals, while in rice grains results agree that N and P amount decreased in response to ozone, while Mn and Cu content increased, and Na was not changed.

Table 5: Effect of ozone stress on the mineral content of major crops.

Minerals	**N**	**P**	**K**	**Ca**	**Mg**	**Na**	**Fe**	**Mn**	**Zn**	**Cu**	**B**		
Crop species	**Ozone effect**											**Organ**	**References**
Corn, *Zea mays* L.	nd	–	–	nd	↓	nd	↑	–	↑	↑	nd	Seed	Garcia *et al*. (1983)
Ladino clover, *Trifolium repens*	–	–↑	–↑	–	–	↓	↑	–↑	–	–↑	–	Stalk + leaf	Blum *et al*. (1982)
Mustard, *Brassica campestris* L	nd	–	–↓	–↓	–↓	nd	nd	nd	–↓	nd	nd	Seed	Singh *et al*. (2009)
Potato, *Solanum tuberosum* L.	nd	–	–	↑	–↓	nd	–	–	–	nd	nd	Stalk + leaf	Fangmeier *et al*. (2002)
Potato, *Solanum tuberosum* L.	–↑	–	–	–	–	nd	↓	–↑	–	nd	nd	Tuber	Fangmeier *et al*. (2002)
Rice, *Oryza sativa* L.	↓	↓	↓↑	–↓↑	↓↑	–	–↓	↑	–↑	↑	nd	Seed	Rai *et al*. (2010), Frei *et al*. (2012), Wang *et al*. (2012), Zheng *et al*. (2013)
Sweet Potato, *Ipomoea batatas* (L.) Lam.	↑	–	–	↑	↑	nd	nd	nd	nd	nd	nd	Tuber	Keutgen *et al*. (2008)
Wheat, *Triticum aestivum* L.	nd	↑	↑	–↑	↑	–	–	↑	↑	↑	nd	Seed	Fuhrer *et al*. (1990), Pleijel *et al*. (2006), Feng *et al*. (2008), Zheng *et al*. (2013)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on minerals content. Minerals not determined (nd).

Garcia *et al.* (1983) found that the seed of maize increased micronutrients (Fe, Zn and Cu), whereas the macronutrients decreased or not changed due to ozone exposure. In mustard Singh *et al.* (2009) observed that in exposed plants the levels of Ca, K and Mg decrease with normal application of NPK, but when 1.5x the normal NPK application was added the Ca, Mg and K concentrations was not modified in the presence of ozone.

4.6. Physical and sensory aspects

Physical aspects such as mass, size, shape, visual damages, texture and flavor are also important in determining the quality of marketable crop products, especially in horticultural products. Symptoms of visible injury appear typically in the leaves, consequently leafy vegetables become highly susceptible to toxic effects of ozone. In broad-leaved plants, the symptoms due to acute exposure include bleaching (small unpigmented necrotic spots), flecking (small brown necrotic areas fading to grey or white), stippling (small punctuate spots which may be white, black or red) and bifacial necrosis (when the entire tissue through the leaf is killed developing a range of color from white to dark orange-red). Whereas, injuries attributed to chronic exposure appear as chlorosis (yellowing due to the chlorophyll breakdown) and bronzing (red-brown pigmentation induced by phenylpropanoids accumulation) (Iriti and Faoro, 2008; Krupa *et al.*, 2001). In a study with spinach and lettuce exposed to elevated ozone (NFCs+ozone) visible foliar injury symptoms in the form of blackish and necrotic bifacial lesions where mainly observed in the interveinal and marginal area of mature leaves (Calatayud *et al.*, 2003, 2002). Temple *et al.* (1990) reported that lettuce and onions chronically exposed to ozone exhibited severe leaf injury, while no injury symptoms could be noticed observed in broccoli. These visible injuries are particularly undesirable when the marketable value of the crop depend on the appearance especially because it can cause an obvious loss of economic crop value.

In some cases flavor may be also modified by changing compounds profile. The soluble solids content (an indirect measurement of sugar content, i.e. sweetness) of watermelon fruit was decreased from 4 to 8% due to exposure to ambient levels of ozone, leading to decrease in fruit quality (Gimeno *et al.*, 1999). Ozone also tended to reduce the sweetness index in two different cultivars of strawberry (Keutgen and Pawelzik, 2008). In potato (cv. Cherokee) exposed to ozone, Pell and Pearson (1984) observed an increase in total glycoalkaloids, which may be lead to bitterness. In addition, another study with potato plants exposed to ozone in open-top chambers found that paste from tubers was more viscous under elevated ozone in one year (1998) and starch granules were more resistant to swelling under elevated ozone in the following year (1999) (Donnelly *et al.*, 2001).

Chalk is an opaque area in the rice grain and is an important quality characteristic in rice. Chalk areas are undesirable because it changes rice cooking and appearance, which negatively affects rice quality. Moreover, chalky grains tend to be

weaker and break easily, thus decreasing mill yield (Wang and Frei, 2011). Wang *et al.* (2012) showed that chalky grain percentage was higher due to ozone exposure, while chalkiness area and chalkiness degree remaining unchanged. Furthermore, they observed that long-term ozone exposure increased surface firmness and reduced acceptability of cooked rice. The study suggested the starch in rice grain grown in high ozone levels exhibited lower viscosity and elasticity.

4.7. Feed value of forage for ruminant animals

Forage quality is determined by its digestibility, nutrient content (proteins, lipid, sugars, starch, minerals) and anti-nutrients content (Vandermeiren and Pleije, 2011; Waghorn and Clark, 2004). Generally, ozone decreases forage quality and it can lead to secondary effects such as lower milk and meat production from grazing animals (Vandermeiren and Pleije, 2011), and thus it is possible to link ozone with indirectly impairment on food security.

Digestibility and protein content are the most studied aspects regarding the impacts of effect in forage quality. Fiber fractions (neutral detergent fiber, NDF; acid detergent fiber, ADF; lignin) and phenolic content are important parameters used to determine the plant material digestibility and, commonly, these two parameters are inversely correlated with forage digestibility (Wang and Frei, 2011). Analyzing the Tabla 6 it is notable that ozone decreased leaf digestibility (Table 6), whereas fiber content are increased (Table 3) in vast majority of cases. The ADF fraction and lignin is inversely correlated to forage digestibility, while NDF is more closely associated with voluntary forage intake than with digestibility (Jung and Allen, 1995).

In clovers grown under ozone exposure, Muntifering *et al.* (2006) observed a decrease in IVDMD (in vitro dry-matter digestibility), in IVCWD (in vitro cell-wall digestibility) and lignin, but no differences in NDF (neutral-detergent fibre) and soluble phenolics concentration were reported. Similarly, Gonzalez-Fernandez *et al.* (2008) observed a negative impact on clover forage grown under ozone, and NDF and lignin enhanced while IVDMD decreased. In a study performed by Gilliland *et al.* (2012), a mixture grassland species (*Lolium arundinacea, Paspalum dilatatum, Cynodon dactylon and Trifolium repens*) exposed to twice (2x) ambient ozone concentration contained approximately 8% more NDF and 15% greater concentration of soluble phenolics than forage grown in non-filtered air (NF), but concentrations of ADF and lignin of forage were approximately equal. In addition, the authors fed white rabbits (*Oryctolagus cuniculus*) with these two forages and observed that the digestibility was 5.5 g per day greater for rabbits that ingested the NF, than the forages grown under 2x ambient ozone. The nutritive quality of little bluestem and sericea lespedeza exposed to ambient and 2x ambient ozone concentration were respectively decreased in 2% and 7%, and the authors explain that as a result of increased levels of cell wall constituents and decreased in vitro digestibility (Powell *et al.* 2003).

Table 6: Effect of ozone stress on the digestibility of forage crops for ruminant herbivores.

	Crop species	Organ	Ozone effect	References
Digestibility	Clover, *Trifolium* spp.	Leaf	↓	Muntifering *et al*. (2006), González-Fernández *et al*. (2008)
	Grassland species mixture	Leaf	↓	Gilliland *et al*. (2012)
	Lespedeza, *Lespedeza cuneata*	Leaf	– / ↓	Powell *et al*. (2003)
	Little bluestem, *Schizachyrium scoparium*	Leaf	– / ↓	Powell *et al*. (2003)
	Poa pratensis L.	Leaf	↓	Bender *et al*. (2006)
	Rice straw, *Ozyza sativa* L.	Leaf	↓	Frei *et al*. (2011)

Ozone stress increase (↑), decrease (↓) or does not show significant difference (–) on digestibility.

In *Poa pratensis*, a high-yielding perennial pasture grass in Europe, early-season ozone exposure caused a loss in the relative feed value of 8%, which is enough to have nutritional implications for herbivores utilization, whit consequences in voluntary intake and digestibility (Bender *et al.*, 2006). Frei *et al.* (2011) studied the effect of ozone on the nutritive quality of rice straw, a by-product of rice grain with important feed value for ruminant livestock. The effects of ozone on the chemical composition of straw were clearly dependent on the ozone level, with significant changes even at ambient ozone concentrations. Increases in crude ash, lignin and phenolics concentration adversely affected the digestibility as demonstrated in incubation experiments simulating rumen digestion *in vitro*. Taking all these studies together, it is possible to note that ozone-induced changes in foliar chemistry can drive alterations in forage quality, which has severe economic and nutritional implications for their utilization by ruminant herbivores.

5. Conclusions

To further understand the dynamic interactions between ozone, plant development and carbon allocation are especially important in a scenario where future ozone levels are predict to increase. Together with seasonal rising temperatures and CO_2 concentrations, ozone exposure changes the timing of carbon dynamics in plants with major detrimental impacts of crop growth rates and seeds development, increasing stress among plants (Fuhrer, 2009; Long *et al.*, 2005). The metabolic switch elicited in plants exposed to medium to elevate ozone levels might lead to 'hidden' changes in qualitative and nutritional properties of natural products, with an over-all risk and consequences on food and feed chain. After analyzing numerous works performed around the world with different plants and distinct crop varieties, we can conclude that the negative impacts of ozone need to be considered in a combination of yield and quality parameters. Changes associated with secondary metabolites biosynthesis can be detrimental for the plant's fitness, and when we consider the allocation costs, modifications of food value and composition could possibly be more significant than biomass yield reductions alone in the assessment of ozone effects (Bender and Weigel, 2011; Fuhrer and Booker, 2003; Vandermeiren and Pleije, 2011).

Besides, some quality aspects of crops such as enhanced seed protein content and secondary metabolites are apparently improved by ozone, and as suggested by Iriti and Faoro (2009), it may be favorable in crops and plants that provide foodstuffs and beverages enriched of bioactive phytochemicals. Even so, reports concerning the effects of chemical altered marketable crops are still lacking, and the consequences for human nutrition need to be studied in more detail. Future challenges thus include mitigation of ozone-induced changes and development of ozone tolerant crops, especially in regions where agroecosystems are presented as key strategy to sponsor communities' food supply in attempt to avoid further ecological impacts and to improve the quality of the agricultural products consumed by us.

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2. JUSTIFICATIVA

Apesar de serem nativas da América, os maiores consumidores e produtores das pimentas *Capsicum* são China, India e México, os quais são países em desenvolvimento, onde é previsto que as concentrações de O_3 continuem aumentando (IPCC, 2001; Vingarzan, 2004; Dentener et al., 2005; FAO, 2010; Avnery et al., 2011b; Bosland e Votava, 2012). Estudos têm mostrado que a alta concentração de ozônio nestes países tem causado sérios danos a diversas cultivares e, assim, afetado negativamente a produção agrícola das mesmas. Porém, pouco se sabe sobre os efeitos deste poluente em plantas do gênero *Capsicum*, principalmente em países latino-americanos (Tiwari et al., 2005; Wang et al., 2007). Além disso, só recentemente tem se avaliado as alterações na qualidade dos produtos agrícolas de plantas expostas a estresses ambientais, como O_3, e pouco se sabe sobre os efeitos do ozônio nos parâmetros de qualidade e metabolismo secundário de frutos de pimentas.

3. OBJETIVOS

Gerais:

O objetivo do presente trabalho foi avaliar os efeitos do ozônio tanto no tecido foliar quanto no fruto das plantas *C. baccatum* L. var. *pendulum*.

Específicos:

1. Realizar uma análise integrada dos aspectos fisiológicos, bioquímicos e parâmetros de estresse oxidativo relacionados à toxicidade do ozônio sobre a planta *C. baccatum L. var. pendulum*.

2. Avaliar o efeito da exposição ao ozônio sobre o conteúdo de fenólicos, de capsaicinóides e de carotonóides, bem como o potencial antioxidante e potencial inibidor de lipoperoxidação de frutos da pimenta *Capsicum baccatum* L. Var. *pendulum* acesso pimenta-dedo-de-moça.

4. RESULTADOS

Os resultados desta Dissertação estão separados em duas partes: um artigo publicado que será apresentado nesta seção e resultados ainda não publicados que estão em anexo como resultados complementares.

Artigo

“Effects of chronic elevated ozone concentration on the redox state and fruit yield of red pepper plant *Capsicum baccatum*”

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Effects of chronic elevated ozone concentration on the redox state and fruit yield of red pepper plant *Capsicum baccatum*

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ABSTRACT

Ozone (O_3) is one of the most harmful air pollutants to crops, contributing to high losses on crop yield. Tropospheric O_3 background concentrations have increased since pre-industrial times reaching phytotoxic concentrations in many world regions. *Capsicum* peppers are the second most traded spice in the world, but few studies concerning the O_3 effects in this genus are known. Thereby, the aim of this work was to evaluate the effects of chronic exposure to elevated O_3 concentrations in red pepper plant *Capsicum baccatum* L. var. *pendulum* with especial considerations on the leaf redox state and fruit yield. Fifteen *C. baccatum* plants were exposed to O_3 in open-top chambers during fruit ripening (62 days) at a mean concentration of 171.6 $\mu g/m^3$ from 10:00 am to 4:00 pm. We found that O_3 treated plants significantly decreased the amount and the total weight of fruits, which were probably a consequence of the changes on leaf oxidative status induced by ozone exposure. Ozone exposed plants increased the reactive oxygen species (ROS) levels on the leaves, which may be associated with the observed decrease on the activity of enzymatic antioxidant defense system, as well with lower levels of polyphenol and reduced thiol groups. Enhanced ROS production and the direct O_3 reaction lead to biomacromolecules damages as seen in the diminished chlorophyll content and in the elevated lipid peroxidation and protein carbonylation levels. Through a correlation analysis it was possible to observe that polyphenols content was more important to protect pepper plants against oxidative damages to lipids than to proteins.

1. Introduction

Ozone (O_3) is a gas naturally find in both troposphere and stratosphere. Tropospheric O_3 generation is based on a complex series of photochemical reactions from primary pollutants mainly generated by the human activities, such as methane (CH_4), carbon monoxide (CO), nitrogen oxides (NOx) and volatile organic compounds (VOCs), and sun light (IPCC, 2001). In the stratosphere, O_3 has a role of filtering the ultraviolet rays, whereas in the troposphere it exerts harmful effects on living organisms due to its strong oxidizing potential (+2.07 eV). When absorbed by animals and plants this pollutant is capable of reacting with different biomacromolecules and generate various reactive oxygen species (ROS). Therefore, it is well-established that O_3 may directly or indirectly lead to oxidative damages to lipids, proteins and nucleic acids, which might affect the key cell functions and plant physiological processes (Halliwell and Gutteridge, 2007; Iriti and Faoro, 2008).

In plants, O_3 penetrates leaves through the stomata where it can be dissolved in the apoplastic fluid. Once inside the substomatal chamber, O_3 can be spontaneously decomposed into ROS or reacts with a number of compounds present in the cell wall, apoplastic fluid and plasma membrane to form ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl radicals ($OH^{\bullet}$). However, ROS are also normal byproducts physiologically generated from various sources during cell metabolism, and for this reason plants have evolved very efficient enzymatic and non-enzymatic antioxidant systems, responsible for maintaining the baseline levels of ROS (Halliwell and Gutteridge, 2007; Castagna and Ranieri, 2009). The antioxidant enzyme SOD, which is found in almost all cellular compartments, makes the dismutation of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide

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(H_2O_2). CAT is primarily found in peroxisomes and converts H_2O_2 into O_2 and water, whereas the APX is thought to be the most important H_2O_2 scavenger operating in chloroplasts and uses ascorbic acid as a reducing substrate (Halliwell and Gutteridge, 2007).

Enzymatic ROS scavenging mechanisms in plants include different enzymes namely the superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). Superoxide dismutase, which is found in almost all cellular compartments, acts as the first line of defense against ROS dismutating superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), while APX, GPX, and CAT subsequently detoxify H_2O_2 to form harmless end products. CAT is primarily found in peroxisomes and converts H_2O_2 into O_2 and water, whereas the APX is thought to be the most important H_2O_2 scavenger operating in chloroplasts and uses ascorbic acid as a reducing substrate. Besides these enzymes, cells possess a number of non-enzymatic antioxidant compounds with high reducing power such as ascorbic acid, glutathione, tocopherols and a variety of phenolic compounds, which are also responsible for detoxifying ROS. Both enzymatic and non-enzymatic antioxidant defense systems play a fundamental role to maintain the cellular redox balance, preventing that overproduced ROS remain unscavenged which could lead to irreparable metabolic dysfunction and cell death (Noctor and Foyer, 1998; Blokhina et al., 2003; Halliwell and Gutteridge, 2007; Gill and Tuteja, 2010; Sharma et al., 2012).

Some of the negative effects of O_3 on plants include photosynthesis reduction, water loss, leaf chlorotic and necrotic spots, accelerated senescence, flower injury, pollen sterility, and diminished yield (Cho et al., 2011; Wilkinson et al., 2012). For these reasons, tropospheric O_3 has been considered the most damaging air pollutant to crops, leading to adversely economic impacts on agriculture (Heagle, 1989; Ashmore, 2005). According to Avnery et al. (2011a) the global yield reductions due to O_3 exposure in 2000 ranged from 8.5 to 14 percent, 3.9 to 15 percent and 2.2 to 5.5 percent, respectively for soybean, wheat and maize, while the global crop production losses in the same year were estimated to be 79–121 million metric tons worth US$ 11–18 billion.

Due to the emission of the primary precursors, from fossil fuels burning, biomass burning and industrial emissions, the global O_3 background levels increased 36 percent since pre-industrial time. Furthermore, projection studies indicate that average global surface O_3 concentrations are expected to rise significantly throughout the 21st century (values ranging from 84 to 168 $\mu g/m^3$) especially in developing countries resulting from rapid industrialization (IPCC, 2001; Vingarzan, 2004; Dentener et al., 2005; Avnery et al., 2011b). It suggests that current and future O_3 impacts on crops and forests in these countries are expected to be very significant (Emberson et al., 2001; Ashmore, 2005).

The fruits of *Capsicum* genus plants, commonly known as red pepper, chili pepper or *Capsicum* pepper, are worldwide used as food and spice to enhance food taste and to impart color, pungency (heat) and particular flavor to food (De, 2003). *Capsicum* peppers are also a remarkable source of health-promoting compounds such as vitamins, carotenoids, capsaicinoids and phenolics, which present well-known antioxidant properties (Edge et al., 1997; Rice-Evans et al., 1997; Oyagbemi et al., 2010; Wahyuni et al., 2011). Moreover, *Capsicum* peppers are the second most traded spice in the world, second only to *Piper* peppers (Weiss, 2002), and their annual production has increased substantially over the past few years (Bosland and Votava, 2012). The most important chili pepper producers and exporters include China, India and Mexico (FAO, 2010), which are developing countries where, as aforementioned, the O_3 concentrations are expected to rise more than other countries. For these reasons chili peppers can be regard as an interesting research target to evaluate the negative effects of O_3.

It is commonly found on the literature studies that describe the effects of low O_3 concentration in a long-term period ($< 80\ \mu g/m^3$ – chronic exposure) or high O_3 concentration in a short-term period ($> 160\ \mu g/m^3$ – acute exposure) in plants (Krupa et al., 2001). However, studies showing the effects of high O_3 concentration exposure in a long-term period are still scarce. Since O_3 concentration has been increasing worldwide achieving mean concentrations above 160 $\mu g/m^3$ in many world regions during the growing season of important cultivars (Avnery et al., 2011a), and taking into account that projections for 2030 show that more regions will reach these high O_3 concentrations (Avnery et al., 2011b), it is important to understand how this condition affects the plants physiology and biochemistry. Therefore, the aim of present work was to evaluate how red pepper plants *C. baccatum* L. var. *pendulum* are affected by chronic exposure to elevated O_3 concentrations, mainly regarding the foliar redox state and fruit yield.

2. Material and methods

2.1. The open-top chambers construction

Based on the model developed by Heagle et al. (1973), and improved by Aidar et al. (2002), six open-top chambers (OTCs) were built. Chambers were 106 cm high and 90 cm diameter. Ambient air was introduced in the chambers driven by a 30 cm Ø 1/6 hp fan connected on the bottom side of each chamber. In three chambers an ozonizer (OZ Engenharia, model GHR150B, Porto Alegre, Brazil) based on corona effect and supplied with ambient air was connected.

2.2. Cultivation conditions

Seeds of *Capsicum baccatum* L. var. *pendulum* accession "Pimenta-dedo-de-moça" were obtained from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária – Brazilian Agricultural Research Corporation). The seeds were sown in pots containing standardized substrate (vermiculite, peat moss and medium washed sand in the ratio 2:1:1) and grown in a greenhouse (at Center of Ecology of the Federal University of Rio Grande do Sul – UFRGS) under a shade mesh (cutting 30 percent radiation) and kept away of known sources of air pollutants. The pots were watered twice a day (7:00 am and 5:00 pm) for 10 min with tap water through an automatic irrigation sprinkler system until the complete development of plants (75 days). During this period each pot additionally received 100 mL of fertilizer solution (4 g/L of Vitaplan Nutriverde 13-13-15 + Micronutrients – Nutriplan Products Company, Paraná, Brazil) at two time points (25 and 50th day of growth).

2.3. Ozone fumigation

After the complete development of plants, 30 plants were separated in six OTCs (five plants per chamber). Half of them were subjected to O_3 fumigation and the other half was grown at unpolluted local atmosphere. The OTCs were located outdoors fenced in an area of 9 m^2 lined with shade mesh (cutting 30 percent radiation), next to the Center of Ecology at UFRGS. A continuous drip irrigation system was used throughout the experiment to ensure adequate and homogeneous water availability for all plants. The O_3 exposure in the OTCs was carried out during the summer of 2012 (February – March). Ozone fumigation occurred during 62 days, from Monday to Saturday from 10:00 am to 4:00 pm because this period of day is considered to be the peak of O_3 production found under ambient conditions. During the experimental period, the meteorological data of temperature and relative humidity within the exposure chambers were collected using a data logger. The temperature ranged from 21.8 to 39 °C (mean = 32 ± 3.99 °C) and relative humidity from 53 to 95.25 percent (mean = 70.3 ± 8.98 percent), with an average photoperiod of approximately 13 h. The monitoring of O_3 concentration inside the chambers (control and ozone treatment) was performed once a week by the colorimetric method described by Apha (1992). Gases were sampled in a fresh washer of impinger type, containing 70 mL of absorbent solution of potassium iodide two percent with the help of a gas sampler (LaMotte, model BD, Chestertown, USA) at a flow of 1.5 L min^{-1}. In the control OTCs none O_3 could be detected, while in the OTCs coupled to an ozonizer the O_3 concentration ranged from 125.9 to 251.8 $\mu g/m^3$ (mean = 171.6 $\mu g/m^3$ ± 44.79).

2.4. Sample preparation and analysis

After 62 days of O_3 exposure all leaves from 2.5 to 10 cm length were collected, washed with distilled water and the surface moisture was wiped out. Foliar tissue samples (ten discs of 0.30 cm^2 size) were obtained from pooled leaves from the same plant. This procedure was performed with all plants (fifteen controls and fifteen O_3 treated). All samples were immediately frozen, with the exception of the

discs used for chlorophyll analysis. All spectrophotometric analyses were conducted on a spectrophotometer (SpectraMax® 190 UV–vis Microplate Reader; Molecular Devices, CA, USA).

2.4.1. Fruits yield assessment

The number of pepper fruits was weekly conferred to account for the losses of fruits. After 62 days of O_3 exposure, the fruits were collected, counted, weighed and separated in immature (green fruits) and mature (red fruits) peppers, for each plant. Since the mean weight of green and red fruits showed no difference, the green fruits were also used in subsequent analysis.

2.4.2. Chlorophyll content

Analysis for the determination of chlorophyll pigment content in leaves was carried out on samples containing ten foliar discs taken from different leaves from the same plant. The discs were incubated with ethanol (96 percent) during 15 days in darkness to extract the pigments. The absorbance of leaf pigment extracts was measured at 665 and 649 nm. Concentrations of chlorophyll a and b, were calculated according to extinction coefficients and equations reported by Knudson et al. (1977). The results were expressed as mg Chlorophyll/g dry weight.

2.4.3. Total phenolic content assay

The leaf total phenolic content was determined by the Folin–Ciocalteu assay (Singleton and Rossi, 1965). For each plant, ten leaf discs were homogenized in 1 mL of 100 mM sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4) pH 7.6, followed by centrifugation at 3000 × *g* during 10 min at 4 °C. The supernatant was mixed with trichloroacetic acid (TCA) ten percent final concentration, and centrifuged at 12,000 × *g* during 10 min at 4 °C. The samples and phenolic standard (tannic acid: 0, 2.5, 5, 10, 20 mg/L) was mixed with Folin–Ciocalteu 1 N reagent and aqueous sodium carbonate (35 percent). Total phenolic content was determined colorimetrically at 725 nm and expressed as μg of tannic acid equivalents (TAE) per μg of protein.

2.4.4. Antioxidant enzyme assays

For the determination of antioxidant enzymes activity, ten leaf discs per plant were homogenized in 2 mL of 50 mM sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and five percent polyvinylpyrrolidone (PVP), pH 7.8. The homogenate was centrifuged at 13,000 × *g* for 10 min at 4 °C. Supernatant was used for enzyme activity and protein content assays. All steps in the preparation of the enzyme extract were carried out at 4 °C.

Catalase (CAT, EC 1.11.1.6) activity was measured according to Aebi (1984) by monitoring the rate of decrease in H_2O_2 absorbance at 240 nm. For the CAT assay the reaction mixture contained 25 mM (pH 7.0) sodium phosphate buffer, 25 mM of H_2O_2 and enzyme extract. The H_2O_2 was quantified by its molar extinction coefficient (36 $M^{-1}.cm^{-1}$) and the results were expressed as CAT units/mg of protein (One unit of CAT was defined as the enzyme amount necessary to reduce 1 mM of H_2O_2 per minute at 28 °C).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbic acid, 1 mM H_2O_2, and enzyme extract. The decrease in absorbance at 290 nm during 1 min was recorded and the amount of oxidized ascorbate was calculated using the extinction coefficient (E= 2.8 $mM^{-1}.cm^{-1}$). The results were expressed as APX units/mg of protein (One unit of APX was defined as the enzyme amount necessary to oxidize 1 mmol.mL^{-1} of ascorbate per minute at 28 °C).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed according to Beyer and Fridovich (1987). The assay is based on the riboflavin activation by a photon, thereby being reduced to a semiquinone which leads to a subsequent reduction of O_2 to O_2^- which, in turn, reduces nitro blue tetrazolium (NBT) to insoluble purple formazan. The formazan is read at 560 nm. The reaction mixture contained enzyme extract, 0.04 mM riboflavin and 10 mM sodium phosphate buffer (pH 7.8) containing 0.056 mM NBT and 10 mM methionine, 0.025 percent Triton-X 100. This mixture was exposed at 60 W fluorescent light for 10 min. One unit of SOD was defined as the amount that causes an inhibition of 50 percent in the NBT reduction. The results were expressed as SOD units/mg of protein.

2.4.5. Measurement of reactive oxygen species levels

For ROS measurements (Babu et al., 2003) ten leaf discs per plant were homogenized in 1.5 mL of Tris–HCl 10 mM pH 7.3 and centrifuged at 15,000 × *g* for 10 min at 4 °C. ROS production was assayed by mixing samples supernatants in a ratio 1:1 with 20 μM 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCFDA) solubilized in dimethyl sulfoxide (DMSO). The mixture was incubated in the dark for 10 min at room temperature. The H_2DCFDA is a non-fluorescent compound that is cleaved by endogenous esterases present inside the cells. The acetate-free, reduced form of 2,7- dichlorodihydrofluorescein (H_2DCF) can be oxidized by ROS to form a highly fluorescent compound, 2,7- dichlorofluorescein (DCF). The DCF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a fluorometer (SpectraMax M5 model; Molecular Device, CA, USA). The results were expressed as relative fluorescence units (RFU)/mg protein.

2.4.6. Thiobarbituric acid reactive substances (TBARS) assay

The TBARS method (Hodges et al., 1999) was used to measure malondialdehyde (MDA) levels, which is a secondary end product of the polyunsaturated fatty acids oxidation. For each plant, ten leaf discs were homogenized in 1 mL of ethanol:water (80:20, v:v), followed by centrifugation at 3000 × *g* for 10 min at 4 °C. The supernatant was mixed with twenty percent TCA (1:1), and centrifuged at 12,000 × *g* for 10 min at 4 °C. An aliquot of appropriately diluted sample was added to a test tube with the same volume of either (i) thiobarbituric acid (TBA) solution (0.67 percent), or (ii) water. Samples were then mixed vigorously, heated at 95 °C for 30 min, cooled and read at 440 nm, 532 nm, and 600 nm. Malondialdehyde equivalents were calculated with the following formula:

(1) $[(Abs\ 532_{TBA} - Abs\ 600_{TBA}) - (Abs\ 532H_2O - Abs\ 600H_2O)] = A$
(2) $(Abs\ 440_{TBA} - Abs\ 600_{TBA})\ 0.0571) = B$
(3) $[(A - B)/157\ 000]\ 10^6$ = MDA equivalents (nmol. mL^{-1})

The results were expressed as MDA equivalents (nmol. mL^{-1})/mg protein.

2.4.7. Carbonyl groups determination

The quantification of carbonyl groups was used as a parameter for oxidative damage to proteins. The assay is based on the reaction of carbonyl groups with dinitrophenylhidrazine (DNPH), as previously described by Levine et al. (1990). Proteins from homogenate leaves (Tris–HCl, pH 8.1) were precipitated by the addition of TCA in a final concentration of ten percent. The samples were centrifuged at 4000 × *g* for 5 min at 4 °C, the supernatants were discarded and the pellets were resolubilized in 10 mM DNPH. The mixture was incubated at room temperature for 1 h, followed by re-addition of TCA (ten percent final concentration) and centrifugation at 16,000 × *g* for 5 min at 4 °C. The protein pellets were washed three times with ethanol:ethyl acetate (1:1, v/v) and finally dissolved in 1 mL 8 M urea (pH 2.3). Samples absorbance were read in a spectrophotometer at 370 nm. The carbonyl groups were calculated using an extinction coefficient of 22 $mM^{-1}\ cm^{-1}$. Blank controls without DNPH were performed for each point and its absorbance were discounted from DNPH-incubated samples to ensure the assay specificity for carbonyl groups. Results were expressed as nmol carbonyl/mg protein.

2.4.8. Total reduced thiol groups content

The leaves were homogenized in 100 mM Tris–HCl (pH 8.1) containing EDTA (20 mM), and the homogenates were centrifuged at 12,000 × *g* for 10 min at 4 °C. Total thiols were estimated as described by Ellman (1959) with some modifications. An aliquot of supernatants was mixed with 100 mM Tris–HCl (pH 8.1) and 10 mM 5,5′-dithiobis (2-nitrobenzoic acid) at a ratio of 10:10:1. The color was allowed to develop for 1 h, and the absorbance was measured at 412 nm. Total reduced thiol groups content was calculated using an extinction coefficient of 14.15 $M^{-1}\ cm^{-1}$. Results were expressed as nmol SH/mg protein.

2.4.9. Anthocyanins content

Anthocyanins were measured according to the procedure based on the methods of Rabino and Mancinelli (1986) and Feinbaum and Ausubel (1988). Total plant anthocyanins pigment were extracted from ten leaf discs per plant in 1.5 mL of methanol:HCl (99:1) and 1 mL of distilled water. The samples were centrifuged at 16,000 × *g* for 15 min at 4 °C. One milliliter of supernatant was added to 1 mL of chloroform to separate chlorophylls from anthocyanins. The levels of anthocyanin pigments were determined by spectrophotometric measurements of the aqueous/methanol phase. The absorbance of the extracts was measured at 530 (peak of absorption of anthocyanin) and 657 nm. The equation $A_{530\text{-}657}$ was used to compensate the contribution of chlorophyll derivatives to absorption at 530 nm; the corrected A_{530} values provide an estimate of anthocyanin content. The values were normalized by the dry weight (DW) of each sample. Results were expressed as A_{530}/g DW.

2.5. Protein determination

Protein content was measured spectrophotometrically at 595 nm by the protein-dye binding method of Bradford (1976), using bovine serum albumin as standard.

2.6. Statistical analysis

Results were expressed as mean ± SEM and were analyzed using GraphPad Prism version 5.01 (Graphpad Software Inc 2007). The Student's t-test was used to assess differences between the two groups and the means were considered different when presenting $p < 0.05$. Correlation analysis between polyphenols content and carbonyl groups or TBARS levels on leaf tissue were assessed using Pearson's correlation analysis.

3. Results

3.1. Antioxidant enzymes activities

Pepper plants subjected to O_3 treatment significantly decreased the activity of all analyzed antioxidant enzymes on leaf tissue (Table 1). The SOD, CAT and APX activities were respectively reduced in 36 percent, 44 percent and 36 percent, suggesting that the antioxidant enzymatic system was impaired by the O_3 exposure which might have contributed to increase the levels of accumulated leaf ROS.

3.2. Reactive oxygen species generation

The H_2DCFDA is oxidized by ROS present in the tissues homogenate to form a fluorescent compound, DCF. The level of fluorescent compound formed is dependent on the amount of ROS. Plants exposed to O_3 increased DCF relative fluorescence (174.7 percent) as compared to control plants (Table 2), indicating that O_3 exposure leads to ROS accumulation inside the foliar tissue.

3.3. Oxidative damages to protein and lipids

Lipid peroxidation and protein carbonylation on leaf tissue were both higher (56.8 percent and 160.4 percent, respectively) in O_3 treated plants when compared to control plants (Table 2). The same pro-oxidant pattern was also observed when assessing proteic and non-proteic reduced sulphur-containing compounds, which were significantly decreased by the O_3 treatment. In the O_3 exposed plants the total reduced thiol groups content in leaves was found to be 75 percent lower than control plants (Table 2).

3.4. Total polyphenol content

We observed that leaf total polyphenol content decreased about 38 percent in O_3 treated plants (Table 2). In order to assess the relationship between total polyphenols and injuries on lipids and proteins a Pearson's correlation analysis was performed. Pearson's correlations showed an inverse (negative) correlation between total polyphenols and TBARS ($r=-0.8196$; $p<0.0001$) or total polyphenols and carbonyl groups ($r=-0.7095$; $p<0.01$) as observed on Fig. 1. These results suggest that total polyphenols content are more related with protection against oxidative damages to lipids than proteins, which might indicate that the maintenance of adequate polyphenols amount can be essential to prevent lipid injuries on the foliar tissue induced by the presence of O_3.

3.5. Pigments content

The pigment levels of leaves were significantly affected by O_3 exposure as observed on Table 3. In O_3 exposed plants chlorophyll *a*, chlorophyll *b* and total chlorophyll content were 39.96 percent, 29.97 percent and 36.63 percent lower than those of the controls, respectively. We observed a decrease in the Chl *a*/*b* ratio, which indicate that Chl *a* is likely more degraded by O_3 than Chl *b*. Otherwise, the leaf anthocyanins content was 63.5 percent higher in O_3 treated plants leaves than in control plants, corroborating the concept that anthocyanins are responsive to oxidative stress (Table 3).

3.6. Yield

The total weight of fruits and the amount of fruit per plant were decreased by O_3 treatment, with respectively values of 61.5 percent and 67.4 percent (Table 4). However, the average weight of fruit did not differ between groups.

4. Discussion

In this work we showed that a series of biochemical and physiological changes can be triggered in *Capsicum baccatum* L. var. *pendulum* plants chronically exposed to high O_3 levels. Ozone penetrates leaves through the stomata, where it can be dissolved in the apoplastic fluid. However, O_3 is virtually undetectable in the apoplast because, immediately after its entry in the substomatal chamber, it is spontaneously decomposed to ROS or reacts with a number of compounds present in the cell wall, apoplastic fluid and plasma membrane to generate ROS (Castagna and Ranieri, 2009). The substomatal ROS generation induced by O_3 aforementioned together with the decrease in antioxidant enzymes activity observed here, may explain, at least in part, the increase in ROS levels observed on O_3 treated plants (Table 2).

The reductions on the antioxidant enzymes activities, SOD, CAT and APX (about 36 percent, 44 percent and 36 percent respectively – Table 1) observed in response to chronic exposure to high O_3 levels, suggests that O_3 exposure is decreasing pepper plants

Table 1
Effects of chronic elevated O_3 concentration exposure on antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX)] of pepper plant leaf tissue. Data are presented as mean ± SEM. The Student's t-test was used to assess differences between control and exposed plants, considering $p<0.05$ as significant.

Enzimatic assays	Units	p value	Control	Ozone exoposed
SOD	(U/mg protein)	<0.01	325.2 ± 28.87 (n=14)	207.0 ± 20.38 (n=15)
CAT	(U/mg protein)	<0.01	39.03 ± 3.51 (n=13)	21.81 ± 3.12 (n=12)
APX	(U/mg protein)	<0.05	12.79 ± 1.73 (n=12)	8.10 ± 0.99 (n=12)

Table 2
Effects of chronic elevated O_3 concentration exposure on redox status indicators [total reduced thiols, DCF (2,7-dichlorofluorescein), total polyphenols, TBARS (thiobarbituric acid reactive substances) and carbonyl groups] of pepper plant leaf tissue. Data are presented as mean ± SEM. The Student's t-test was used to assess differences between control and exposed plants, considering $p<0.05$ as significant.

Redox status parameters	Units	p value	Control	Ozone exoposed
Total reduced thiols	(μmol)	<0.01	14.29 ± 2.08 (n=12)	3.54 ± 1.41 (n=7)
DCF	(RFU[a])	<0.01	3384 ± 918.9 (n=13)	9296 ± 1370 (n=15)
Total polyphenols	(mg TAE[b])	<0.0001	124.9 ± 9.41 (n=13)	76.88 ± 4.88 (n=15)
TBARS	(nmol MDA eq./mL)	<0.01	87.80 ± 12.72 (n=8)	137.7 ± 11.05 (n=9)
Carbonyl	(nmol)	<0.01	2.13 ± 0.38 (n=10)	5.54 ± 1.04 (n=7)

Notes: All the units are normalized by mg of protein.

[a] Relative fluorescence units (RFU).
[b] Tanic acid equivalents (TAE).

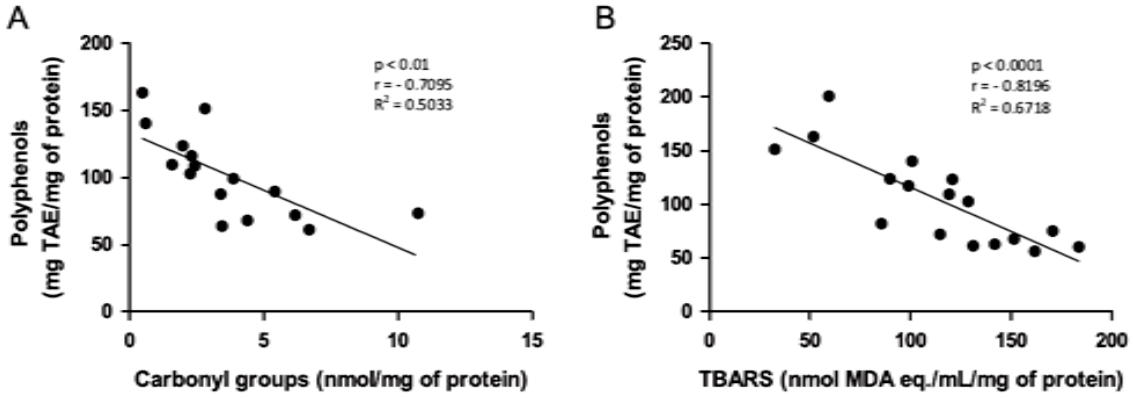

Fig. 1. Pearson's correlation analysis between total polyphenols and carbonyl groups (A) and TBARS (B) from *C. baccatum* L. var. *pendulum* leaves.

Table 3
Effects of chronic elevated O_3 concentration exposure on chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophylls and chlorophyll *a* to *b* ratio of pepper plant leaf tissue. Data are presented as mean ± SEM. The Student's t-test was used to assess differences between control and exposed plants, considering $p < 0.05$ as significant.

Pigments content	Units	*p* value	Control	Ozone exoposed
Chl *a*	(mg)	< 0.0001	7.351 ± 0.425 (n = 12)	4.413 ± 0.226 (n = 14)
Chl *b*	(mg)	< 0.0001	3.686 ± 0.164 (n = 12)	2.581 ± 0.087 (n = 14)
Total chl	(mg)	< 0.0001	11.04 ± 0.587 (n = 12)	6.995 ± 0.312 (n = 14)
Chl *a*/*b*	(mg)	< 0.0001	1.982 ± 0.032 (n = 12)	1.698 ± 0.033 (n = 14)
Anthocyanins	($A_{530-657}$)	< 0.05	4.000 ± 0.193 (n = 12)	6.541 ± 0.931 (n = 14)

Notes: All the units are normalized by g of dry weight.

Table 4
Effects of chronic elevated O_3 concentration exposure on fruit yield of pepper plant *C. baccatum* L. var. *pendulum*. Data are presented as mean ± SEM. The Student's t-test was used to assess differences between control and exposed plants, considering $p < 0.05$ as significant.

Yield indicators per plant	Units	*p* value	Control	Ozone exposed
FTW[a]	(g)	< 0.0001	77.71 ± 7.318 (n = 14)	29.90 ± 5.811 (n = 10)
FAW[b]	(g)	0.1381	17.51 ± 1.049 (n = 14)	15.18 ± 1.033 (n = 10)
FQ[c]	–	< 0.0001	4.500 ± 0.374 (n = 14)	1.467 ± 0.322 (n = 15)

[a] FTW (Fruit total weight).
[b] FAW (Fruit average weight).
[c] FQ (fruit quantity).

capacity to efficiently protect the plants against the toxic effects of ROS. The decline in the APX and CAT activities in treated plants indicates that H_2O_2 levels are increased. Moreover, it has been shown that O_3 might generate H_2O_2 when interacting with cell membrane polyunsaturated fatty acids (Pryor et al., 1991). This fact may also explain, in part, the low SOD activity after the O_3 treatment, once high levels of H_2O_2 are known to inactivate SOD enzyme (Bowler et al., 1992). Furthermore, the diminished SOD, CAT and APX activities may be due to the oxidative damages to leaf proteins, which can be supported by the high levels of carbonyl groups in proteins observed in O_3-treated plants. High levels of carbonyl groups have also been reported for others authors in plants exposed to O_3 (Junqua et al., 2000; Leitao et al., 2008).

Most studies have shown an increase in the activity of APX, SOD and CAT after O_3 exposure (Calatayud et al., 2003; Gillespie et al., 2011; Mishra et al., 2013), but other studies have shown that their activities are dependent on ozone dose and in O_3 high doses may decrease the activity of them (Umponstira et al., 2006; Yan et al., 2010). Some stressors like O_3, aluminum and lead are associated with biphasic responses of different biochemistry parameters, where lower doses are stimulatory and higher doses are inhibitory (Umponstira et al., 2006; Pereira et al., 2010; Wang et al., 2010). Thus, in our work, the observed decrease in the antioxidant enzymes activity and on the phenolic compounds production (Tables 1 and 2, respectively) could be explained by the chronic exposure to high O_3 concentration treatment. However, the effects of O_3 stress on the antioxidant enzymes are very complex and depend on the plant species, treatment intensity and exposure time.

Ozone is considered too reactive to penetrate far into tissues, thereby just a little or none amount of the O_3 is thought to pass unreacted through of the plasma membrane (Pryor, 1992; Castagna and Ranieri, 2009). In membrane cell, the ozone may react with polyunsaturated fatty acids generating ozonide, which under suitable conditions decomposes forming organic radicals, reactive aldehydes and H_2O_2. It is possible that these molecules carry the ozone damage to more distant tissue (Pryor and Church, 1991; Pryor et al., 1991). The H_2O_2 is relatively stable and diffusible (including through cell membranes), compared with many other ROS. Furthermore, H_2O_2 may react with iron and cooper to form OH^*, which is the most reactive oxygen radical known (Halliwell and Gutteridge, 2007). Aldehydes may react with some amino acids leading to damage on proteins (Glomb and Monnier, 1995; Zeng et al., 2006). These peculiarities of ozone prooxidant mechanism may partly explain the increase on lipid peroxidation levels and carbonyl groups content (Table 2) observed in the present work as well as in studies performed with different crops (Junqua et al., 2000; Calatayud et al., 2003; Iglesias et al., 2006; Leitao et al., 2008; Caregnato et al., 2010; Mishra et al., 2013).

Maintenance of cellular thiol redox status is critical for determining protein structure, regulation of enzyme activity, and control of transcription factor activity. Thiol antioxidants act through a variety of mechanisms, especially as components of the general thiol/disulfide redox buffer (Deneke, 2001). According to Mudd et al. (1969) sulfhydryl groups are compounds highly susceptible to oxidation by O_3. Our results show that O_3 treated

plants are in a prooxidative state, since ROS increased and reduced thiol groups levels decreased as may be seen in Table 2. It is known that disturbance in the prooxidant–antioxidant balance, in favor of the former, may lead to the oxidative damages and modulation of redox-sensitive cell signaling pathways (Halliwell, 2006).

Phenolic compounds are secondary metabolites abundant in plant tissues and generally their production is enhanced in stressful situations (Lattanzio et al., 2006), including ozone as showed by Langebartels et al. (1991), Saleem et al. (2001), Frei et al. (2011), Mishra et al. (2013). However differently from literature, our data showed a reduction of these compounds in O_3 treated plants. Two possible explanations for our results are that: (a) the production of phenolic compounds may be more expensive for plants subjected to severe stress because carbon fixation and energy generation probably satisfying just the basic metabolic needs for plant survival, once the photosynthesis process is compromised as suggested by the lower levels of chlorophyll content in treated plants (Table 3); or (b) the O_3 dose was too phytotoxic for pepper plants that it has led to irreversible damages to enzymes related to phenolic compounds synthesis, which could be supported by the protein carbonylation and sulfhydryl oxidation found in O_3 treated plants (Table 2).

Phenolic compounds possess well-known antioxidant properties, which arise from their high reactivity as hydrogen or electrons donors, and from the ability of antioxidant-derived radical to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal ions (Rice-Evans et al., 1997). Another possible mechanism toward phenolics antioxidant activities is their ability to inhibit membrane peroxidation possibly by stabilizing membranes through decreasing membrane fluidity and modifying the lipid packing order (Arora et al., 2000). Havaux and Kloppstech (2001) observed that phenolic compounds-deficient mutant of *Arabidopsis* (tt5) was associated with an increased sensitivity to lipid peroxidation and photosystem destruction when growing under strong light at low temperatures. Foy et al. (1995) showed that O_3 tolerance in different soybeans genotypes is associated with the presence of various kaempferol glycosides. Thus, the lower content of polyphenols observed in our O_3 exposed plants agree with an impairment in the non-enzymatic antioxidant system potentially culminating in the increased macromolecule damage observed in our set of experiments.

Through Pearson's correlation analysis we observed that leaf polyphenols levels were correlated with protection against oxidative damages to lipids ($r=-0.8196$; $p<0.0001$) and to proteins ($r=-0.7095$; $p<0.01$) (Fig. 1). This result suggests that these compounds may be playing an important role especially in protecting against leaf tissue lipid peroxidation, at least under our O_3 exposure condition.

The imbalance in the redox state of treated plants favored the ROS production observed herein, was probably responsible for the degradation of leaf chlorophyll content (Table 3). This effect has been reported in other crops exposed to O_3 such as wheat (Pleijel et al., 2006; Mishra et al., 2013), rice (Sarkar and Agrawal, 2012), beans (Caregnato et al., 2013) and tobacco (Saitanis et al., 2001). The decrease in Chl *a*/*b* ratio in ozonated plants suggests that either Chl *a* was more readily degraded than Chl *b*, or the synthesis of new Chl *a* was reduced by O_3 fumigation. Changes in chlorophyll content are a useful indicator of phytotoxicity induced by O_3 (Knudson et al., 1977; Saitanis et al., 2001). Therefore, the O_3 concentration (171.6 $\mu g/m^3$) obtained in our exposure experiments combined with the exposure time (two months) demonstrated to be highly phytotoxic to *C. baccatum* L. var *pendulum*. Other possible explanation for the observed decrease in chlorophyll content is that the chlorophyll breakdown takes part on the senescence process (Smart, 1994) which is accelerated by O_3 in many plant species (Miller et al., 1999). In our experiment, we observed a decrease of 10–50 percent in the leaves number in O_3 treated plants as compared to two and four percent in control plants after the beginning of O_3 exposure (data not shown).

A common feature of abiotic and biotic stress is the generation of ROS in plant cells (Langebartels et al., 2002). Likewise, anthocyanin synthesis is strongly induced by various stresses e.g. osmotic, UV-irradiation, nitrogen and phosphorus deficiency, low temperature and O_3 (Chalker-Scott, 1999). Purified anthocyanins solutions are four time more efficient as ROS scavengers when compared to ascorbate and α-tocopherol, suggesting that these compounds have a very efficient antioxidant potential (Gould, 2004). In gamma-irradiated *Arabidopsis*, anthocyanin was accountable for 40–50 percent of the total radical scavenging activity (Nagata et al., 2003). Thus, anthocyanins accumulation may be important in O_3 stress tolerance since it may protect plants of injuries due to O_3-induced ROS. Despite the increased content of anthocyanins observed in O_3 treated plants, it seems not to be sufficient to prevent the ROS accumulation and biomolecule damage caused by O_3.

In various environmental stresses, the accumulation of ROS is the major responsible for loss of crop productivity worldwide (Gill and Tuteja, 2010). Therefore, the decrease in fruit yield observed is probably a consequence of the increase in ROS generation and the consequent oxidative damage caused by O_3. Many studies have shown that O_3 decreases the productivity of several different crops (Gerosa et al., 2009; Singh et al., 2009; Sarkar and Agrawal, 2012; Mishra et al., 2013; Zheng et al., 2013). In our work, the plants exposed to O_3 decreased the fruits total weight, which was a consequence of the decrease in the amount of fruits per plant, since the average weight of fruits was not different between controls and O_3-treated plants. Therefore, in some cases O_3 may have prevented the new fruit formation or increased their downfall. Two possible explanations for this result are (a) O_3 directly or via ROS may be inducing fruit downfall, and detrimental effects on flowering and pollen tube extension (Iriti and Faoro, 2008), or (b) O_3-stressed plants could be driving energy to produce normal fruits but in a lower amount.

5. Conclusions

In this study, we deal with the phytotoxic potential of tropospheric O_3 at high concentration for a long period of time. The pepper plants *Capsicum baccatum* L. var. *pendulum* showed a significant decrease in fruits total weight and fruits number, which were probably consequences of the oxidative environment induced by O_3 exposure. The increase of ROS levels on leaves caused by O_3 exposure may be due to the reduction in antioxidant enzymatic defense activity, the diminished polyphenol levels and also the decreased of reduced thiol groups. The increase in anthocyanins levels was not sufficient to prevent ROS generation. Both ROS production and the direct O_3 reaction lead to damages to biomacromolecules as seen in the reduced chlorophyll content and elevation in lipid peroxidation and protein carbonylation levels. Through correlation analysis we observed that polyphenols levels were more correlated with protection against oxidative damages to lipids than to proteins. In sum, this study contributes to understand how elevated O_3 concentrations for long period of time may compromise the integrity of horticultural crops and suggests a potential role of oxidative stress in mediating the O_3-induced impairments on plant with detrimental impacts on agricultural crops productivity.

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5. DISCUSSÃO

Como já mencionado anteriormente, o O_3 é um poluente gasoso com grande potencial oxidante que vem aumentando na atmosfera trazendo, assim, ameaça a saúde de animais e plantas. As plantas são organismos sésseis e por isso são mais vulneráveis aos poluentes (Harborne, 1994). Nas plantas o O_3 entre através dos estômatos e rapidamente se decompõem em várias ERO, dessa forma desencadeando um estado pró-oxidante que levará aos seus efeitos fitotóxicos (Castagna e Ranieri, 2009).

Na literatura a maioria dos trabalhos descrevem os efeitos do O_3 exposto de modo crônico (concentrações abaixo de 40 ppb por longos períodos) ou agudo (concentrações acima de 80 ppb por períodos curtos), porém a concentração de O_3 vem aumentando e em muitos lugares plantas são expostas continuamente a altas concentrações, mostrando a importância de se investigar os efeitos deste poluente quando presente em altas concentrações por longos períodos. Há anos se avaliam as alterações redox e a diminuição da produtividade desencadeadas pelo O_3, mas nos últimos anos tem sido dado, também, atenção a qualidade dos produtos agrícolas derivados de plantas expostas ao O_3. Porém, a maioria destes estudos que avaliam a qualidade é feita com cereais e quase não existem trabalhos com hortaliças, lembrando que as pimentas são o segundo tempero mais consumido e comercializado no mundo. Portanto, neste trabalho foram investigados, em folhas e frutos, os efeitos da exposição crônica de plantas *C. baccatum* a níveis elevados de O_3 durante o período de desenvolvimento dos frutos (62 dias).

Para a realização do trabalho 30 indivíduos da espécie C. baccatum foram colocados em câmaras de topo aberto acopladas ou não a ozonizadores como mostra a Figura 4 localizada nos resultados complementares desta dissertação. Além de ser amplamente utilizado em trabalhos com O_3, este sistema foi escolhido porque permite controlar uma série de variáveis garantindo, assim, que as respostas sejam quase que exclusivamente pela ação do O_3.

Neste trabalho nós mostramos que o O_3 foi capaz de disparar uma série de mudanças bioquímicas e fisiológicas em plantas *Capsicum baccatum* L. var. *pendulum*, quando estas foram expostas cronicamente a altos níveis deste poluente. Além disso, observamos que estas alterações se estenderam aos frutos, culminando em diminuição da produtividade e modificação da qualidade dos mesmos.

Uma das consequências da exposição ao O_3 foi o aumento bastante elevado na produção de ERO no tecido foliar (Tabela 2 - artigo). Além da sabida decomposição do O_3 em outras ERO, este aumento pode ser explicado por uma diminuição na atividade das enzimas SOD, CAT e APX que foi observada neste tecido (Tabela 1 - artigo). A redução na atividade das enzimas antioxidantes SOD, CAT e APX (aproximadamente 36 %, 44% e 36 %, respectivamente) pode ser devido aos danos oxidativos ocorridos nas proteínas, os quais podem ser explicados pelos altos níveis de grupamentos carbonil nas proteínas foliares das plantas tratadas com O_3 (Tabela 2 - artigo), fato frequentemente observado na literatura (Junqua et al., 2000; Leitao et al., 2008). Muitos estudos tem mostrado um aumento na atividade da SOD, CAT e APX após a exposição ao O_3 (Calatayud et al., 2003; Gillespie et al., 2011;

Mishra et al., 2013)(Calatayud et al., 2003; Gillespie et al., 2011; Mishra et al., 2013), enquanto outros tem mostrado que, em altas doses de O_3, estas podem ter sua atividade diminuída (Umponstira et al., 2006; Yan, He, et al., 2010). Da mesma forma que outros elementos tóxicos, como alumínio e chumbo, o O_3 pode apresentar um efeito bifásico em diversos parâmetros bioquímicos (Umponstira et al., 2006; Pereira et al., 2010; Wang et al., 2010; Yan, Chen, et al., 2010), onde baixas doses são estimulatórias e altas doses são inibitórias ou causam danos; a esse efeito se dá o nome de hormesis (Figura 3). Devido a isso é sempre interessante ter uma curva de concentração do elemento tóxico no experimento, o que deve ser objeto de futuros estudos. Por termos apenas uma concentração (dose alta exposta cronicamente) observamos apenas um momento dos efeitos do O_3, os quais consideramos serem os efeitos tóxicos do mesmo. Por conta disso voltaremos a mencionar este fenômeno ao decorrer da discussão.

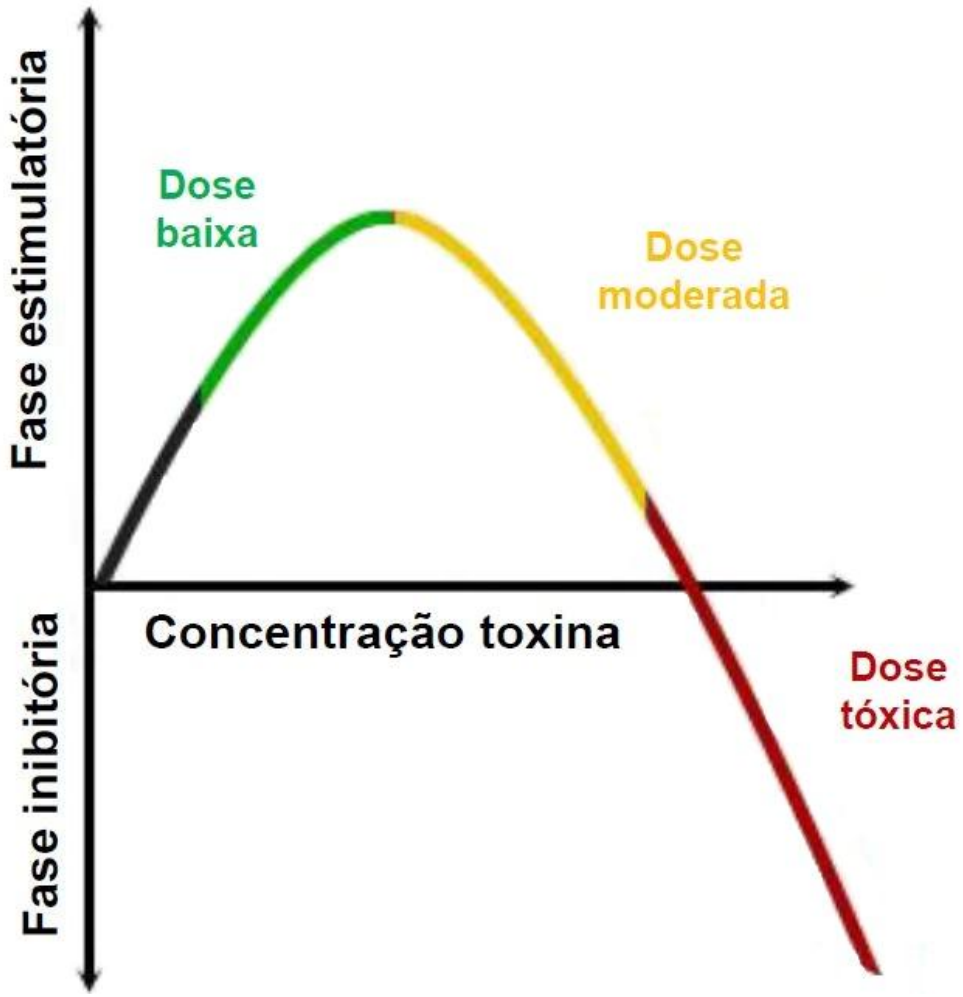

Figura 3: Gráfico mostra uma dose resposta para uma toxina ou estressor geral (por exemplo, exercício físico, álcool, restrição calórica e poluentes) , onde pequenas dose podem ativar mecanismos de reparo (efeito benéfico), enquanto altas doses geralmente inibem ou causam danos (efeito tóxico): fenômeno conhecido como hormesis (Mattson, 2008).

O O_3 é considerado muito reativo para penetrar dentro dos tecidos, portanto somente pouca ou nenhuma molécula de O_3 passa através da membrana plasmática sem reagir (Pryor, 1992; Castagna e Ranieri, 2009). Na membrana plasmática o ozônio pode reagir com ácidos graxos poli-insaturados favorecendo a formação de radicais orgânicos, aldeídos e H_2O_2, os quais possivelmente difundem os danos do O_3 a tecidos mais distantes (Pryor e Church, 1991; Pryor et al., 1991). O H_2O_2 é relativamente estável e difusível pela membrana plasmática, além disso, pode reagir com ferro e cobre para

gerar $OH^{\bullet}$, que é o radical livre mais reativo capaz de atacar qualquer biomolécula (Halliwell and Gutteridge, 2007). Aldeídos podem reagir com alguns aminoácidos levando a danos nas proteínas (Glomb e Monnier, 1995; Zeng et al., 2006). Esta reação em cadeia gerada pelo ozônio pode explicar, pelo menos em parte, o aumento no dano a lipídios e proteínas observado neste trabalho (Tabela 2 - artigo 1), bem como em outros estudos realizados em diferentes culturas (Junqua et al., 2000; Calatayud et al., 2003; Iglesias et al., 2006; Leitao et al., 2008; Caregnato et al., 2010; Mishra et al., 2013).

A manutenção do estado redox de tióis é crítico para manter a estrutura protéica, regular atividades enzimáticas e controlar atividade de fatores de transcrição. Os compostos tiólicos tem propriedades antioxidantes atuando através de vários mecanismos, mas sua principal função é como tampão redox da célula (Deneke, 2001). Dessa forma uma alteração no conteúdo de tiólicos pode indicar se a célula está em um estado pró-oxidante ou antioxidante, além de indicar dano protéico, uma vez que os grupamentos tióis também determinam a estrutura protéica. De acordo com Mudd et al. (1969) os grupamentos tióis são altamente suscetíveis a oxidação por O_3. Nossos resultados mostraram que o tecido foliar das plantas tratadas com O_3 está possivelmente em um estado pró-oxidante, uma vez que as ERO aumentaram e os níveis grupamentos tióis reduzidos diminuíram como pode ser observado na Tabela 2 (artigo). É sabido que um distúrbio no balanço pró-oxidante/antioxidante , favorecendo o primeiro, podem levar a danos oxidativos e possível modulação de vias de sinalização redox-sensíveis (Halliwell, 2006).

Os compostos fenólicos são metabólitos secundários abundantes nas plantas, geralmente apresentam função protetora e por isso sua produção é

reforçada em situações de estresse (Lattanzio et al., 2006), incluindo exposição ao O_3 como mostrado por Langebartels et al. (1991), Saleem et al. (2001), Frei et al. (2011), Mishra et al. (2013). Porém diferente do geralmente encontrado na literatura, nossos dados mostraram uma redução destes compostos nas plantas tratadas. Nós propomos duas possíveis explicações para o nosso resultado, onde uma não necessariamente exclui a outra: a) a produção de compostos fenólicos pode ter um custo mais alto em plantas expostas a estresse severo (como neste estudo), pois a geração de energia e a fixação de carbono provavelmente satisfaçam apenas as necessidades básicas do metabolismo, uma vez que a fotossíntese está comprometida como sugerido pelo baixo conteúdo de clorofila nas plantas tratadas (Tabela 3 - artigo); ou b) a dose de O_3 usada foi fitotóxica (fase inibitória da hormesis - Figura 3) para as pimentas a ponto de causar danos irreversíveis as enzimas relacionadas a síntese de compostos fenólicos, o que poderia ser suportado pelo aumento na carbonilação protéica e oxidação de tióis encontrado nas plantas tratadas (Tabela 2 - artigo).

Compostos fenólicos possuem propriedades antioxidantes bem conhecidas decorrentes de seu potencial doador de hidrogênio ou elétrons, estabilizador de elétrons e quelante de metais de transição (Rice-Evans et al., 1997). Outro possível mecanismo é a sua capacidade de inibir a peroxidação lipídica através de uma estabilização da membrana (Arora et al., 2000). Havaux e Kloppstech (2001) observaram que mutantes de *Arabidopsis* deficientes em fenólicos (tt5) foram mais sensíveis a peroxidação lipídica do que não mutantes quando as plantas foram expostas a forte luminosidade e baixa temperatura. Foy (1995) mostrou que a presença de kaempforol-glicosídeos, em diferentes

genótipos de soja, estão associados à tolerância ao O_3. Em nosso estudo observamos através da correlação de Pearson que, nas folhas, o nível de polifenóis foi correlacionado com a proteção contra dano a lipídios ($r = - 0.8196$; $p < 0.0001$) e proteínas ($r = - 0.7095$; $p < 0.01$) (Figura 1 - artigo). Estes resultados sugerem que estes compostos podem ter papel importante especialmente contra lipoperoxidação no tecido foliar em situações de estresse ou pelo menos sob uma atmosfera rica em O_3. Portanto, o baixo conteúdo de polifenóis encontrado aqui, deve estar relacionado com o aumento no dano a lipídios e proteínas encontrado em plantas tratadas (Tabela 2 - artigo).

Outra consequência da exposição ao O_3 foi a diminuição do conteúdo de clorofila (Tabela 3 - artigo). Este efeito tem sido relatado em vários outros trabalhos com diferentes culturas vegetais expostas ao O_3, tais como trigo (Pleijel et al., 2006; Mishra et al., 2013), arroz (Sarkar e Agrawal, 2012), feijão (Caregnato et al., 2013) e tabaco (Saitanis et al., 2001). A diminuição na razão Chl a/b em plantas ozonizadas sugere que a Chl a foi mais rapidamente degradada do que a Chl b ou teve sua síntese diminuída. Segundo Knudson et al. (1977) e Saitanis et al. (2001), a diminuição no conteúdo de clorofila, em plantas expostas ao O_3, pode ser um indicativo de fitotoxicidade do mesmo. Dessa forma podemos dizer que a concentração de O_3 obtida em nossa exposição (171.6 $\mu g/m^3$ = 85,8 ppb) combinado com o tempo longo de exposição (dois meses) demonstrou-se fitotóxica as plantas *C baccatum*. Outra possível explicação para o observado decréscimo no conteúdo de clorofila é que a degradação da clorofila faz parte do processo de senescência, o qual está acelerado em plantas expostas a ambientes ricos em O_3 (Smart, 1994; Miller et al., 1999). A senescência é um processo de envelhecimento e queda

das folhas, que pode ser observado após o início da exposição, onde contabilizamos uma queda no número de folhas quase 10 vezes maior nas plantas tratadas com O_3 (dados não mostrados).

Uma característica comum tanto do estresse abiótico quanto do biótico é a geração exacerbada de ERO nas células vegetais (Langebartels et al., 2002). Do mesmo modo há nestas situações um aumento na síntese de antocianinas, a qual é um potente antioxidante *in vitro*, chegando a ser até quatro vezes mais eficiente do que ascorbato e *α*-tocoferol (Chalker-Scott, 1999; Gould, 2004). Em *Arabidopsis* expostas a radiação gama as antocianinas foram responsáveis por 40-50% de toda a atividade antioxidante (Nagata et al., 2003). Assim, o acúmulo de antocianinas pode ter um importante papel na tolerância ao estresse por O_3, uma vez que este aumenta a geração de ERO. Porém, o aumento no conteúdo de antocianinas observado nas folhas das plantas ozonizadas (Tabela 3 - artigo) parece não ter sido suficiente para impedir o acúmulo de ERO e os danos às biomoléculas.

Em relação à produtividade dos frutos, observamos que em plantas ozonizadas houve um decréscimo de 61,5% na quantidade de frutos, porém o peso dos frutos separadamente não variou entre plantas tratadas e controles (Tabela 4 - artigo). Além disso, aspectos visuais como forma e cor aparentemente não variaram entre os frutos de plantas ozonizadas e controles. Portanto, em alguns casos o O_3 impediu a formação do fruto ou aumentou a queda dele. Sugerimos duas possíveis explicações para esses resultados: a) ação direta do O_3 ou via ERO podem induzir a queda dos frutos, e efeitos deletérios sobre a floração e tubo polínico (Iriti e Faoro, 2008), ou b) plantas ozonizadas poderiam estar desviando sua energia para produzir poucos frutos,

porém saudáveis. Muitos estudos têm mostrado que o ozônio causa um decréscimo na produtividade de várias culturas vegetais, o qual é acentuado nas espécies mais sensíveis como trigo, soja e tomate (Mills et al., 2007; Gerosa et al., 2009; Singh et al., 2009; Sarkar e Agrawal, 2012; Mishra et al., 2013; Zheng et al., 2013).

Os frutos são os produtos comercializáveis das pimenteiras e, portanto são eles que necessitam apresentar uma boa qualidade para que sua comercialização não seja afetada. Em nosso trabalho, os frutos parecem ter menos danos visíveis do que as folhas uma vez que as folhas apresentaram uma série de alterações, como queda aumentada, clorose e ondulamento das bordas (Figura 4), enquanto os frutos não apresentaram, aparentemente, diferenças visíveis entre tratados e controles. Porém, aspectos visíveis como cor, manchas, formato e peso são apenas um dos parâmetros relacionados à qualidade. A alteração na composição química, devido a situações de estresse, também é algo de grande importância e tem, recentemente, sido discutida como um dos principais aspectos relacionados com a qualidade dos produtos agrícolas (Ashmore, 2005; Wang e Frei, 2011).

Figura 4: imagem A mostra as pimenteiras controles, enquanto a imagem B mostra pimenteiras expostas ao ozônio.

Em relação à mudança de metabolismo podemos dizer que a planta, quando passa por situações de estresse, altera seu metabolismo passando de um metabolismo voltado para o crescimento e reprodução para um metabolismo de defesa, onde a produção de compostos secundários é privilegiada (Iriti e Faoro, 2009). Compostos secundários são os componentes chave do sistema de defesa das plantas atuando na proteção contra uma variedade situações de estresse bióticos e abióticos, tais como herbivoria, infecção microbiana, exposição ao O_3, luz ultravioleta e seca. Geralmente, compostos secundários dependem dos compostos primários como fonte de precursores e é por isso que situações de estresse, como O_3 troposférico, podem levar a um desvio dos recursos disponíveis ao metabolismo de crescimento para o metabolismo de defesa das plantas. Esta alteração, geralmente, acarreta em uma diminuição na produtividade e uma alteração na composição química de folhas, frutos, caules e raízes, consequentemente alterando a qualidade dos produtos agrícolas (Korkina, 2007; Iriti e Faoro, 2009). De acordo com a origem biossintética, os compostos secundários podem ser classificados em três grandes grupos: compostos fenólicos, terpenóides e alcalóides (Croteau et al., 2000). Devido à grande atividade biológica desses compostos, membros destes grupos podem ser tanto benéficos como compostos antioxidantes e anti-inflamatórios quanto danosos como compostos carcinogênicos e tóxicos (Crozier et al., 2006), mostrando a importância de avaliar estes compostos como parâmetros de qualidade em produtos agrícolas.

Em nosso trabalho a exposição ao O_3 mostrou uma alteração em vários fitoquímicos além de alteração no potencial antioxidante de frutos de pimentas.

Dentre os fitoquímicos da pimenta a capsaicina ganha destaque por ser o composto pungente que confere característica aos frutos *Capsicum*. Além disso, se conhece muitas propriedades benéficas desta molécula, como potencial antioxidante, anti-inflamatório, anticancerígeno, analgésico e antiobesidade, dessa forma, conferindo um potencial terapêutico aos frutos de pimenta (Prasad et al., 2006; Kang et al., 2011; Reyes-Escogido et al., 2011). Nas Figuras 7, 8 e 9 podemos ver que a exposição ao O_3 levou a uma diminuição do conteúdo de capsaicina, bem como de outro capsaicinóide - a dihidrocapsaicina, sugerindo uma perda no potencial terapêutico da pimenta.

Além de capsaicinóides, as pimentas também são ricas em carotenóides os quais são os principais metabólitos que conferem cor ao fruto (Lightbourn et al., 2008). Em relação à saúde humana, estes compostos podem prevenir o aparecimento de doenças degenerativas como aterosclerose, câncer e catarata (Edge et al., 1997; Rao e Rao, 2007). Além disso, alguns carotenóides, ao serem absorvidos, podem ser convertidos em vitamina A, o qual é um nutriente essencial e extremamente necessário a vários processos biológicos, incluindo reprodução, sistema imune e diferenciação celular (Wasserman e Corradino, 1971). A fim de avaliar a alteração neste grupo de compostos, quantificamos, por HPLC, 4 carotenóides sabidamente presentes nos frutos de pimentas: zeaxantina, luteína, β-caroteno e α-caroteno (Wahyuni et al., 2011). Como podemos ver nas Figuras 10, 11 e 12, a zeaxantina foi o únicos dos carotenóides que se mostrou diferente entre os grupos, mostrando-se aumentada nos frutos de plantas tratadas. Porém, quando foram mensurados os carotenóides totais foi observado que estes estão aumentados nos frutos tratados com O_3 (Figura 13).

Os compostos fenólicos têm sido os metabólitos mais amplamente estudados em plantas, principalmente em trabalhos que avaliam efeitos de estresse como mostrado na Tabela 4 do referencial teórico, por exemplo. Isto se deve ao fato destas moléculas serem derivadas da via de síntese dos fenilpropanóides, a qual é fortemente responsiva a diversos tipos de estresse ambiental (Korkina, 2007). Corroborando com a literatura, nossos dados mostraram um aumento nos compostos fenólicos totais em frutos de plantas expostas ao O_3 (Figura 14). Estes compostos comumente apresentam propriedades benéficas como potencial antioxidante e anti-inflamatório (Rice-Evans et al., 1996), sugerindo então, que estes frutos possam ter uma qualidade melhor.

De modo geral, uma vez que a planta está sob estresse ela passa a produzir mais compostos de defesa, os quais na maioria das vezes apresentam um grande potencial antioxidante para ajudar a planta a lidar com o estresse (Korkina, 2007). Portanto era esperado que frutos de plantas expostas ao O_3 apresentassem um potencial antioxidante maior. Porém contrariamente ao que se esperava, os frutos de plantas expostas ao O_3 apresentaram um potencial antioxidante mais baixo (Figura 15). Podemos observar também que os extratos dos frutos de pimentas foram capazes de inibir a lipoperoxidação; no entanto não houve diferença entre os frutos de plantas ozonizadas e controles referente a este efeito (Figura 16).

6. CONCLUSÃO

6.1. Geral

Neste trabalho, mostramos que o O_3 desencadeou uma série de alterações deletérias no tecido foliar de plantas *Capsicum baccatum* L. var. *pendulum*, quando estas foram expostas cronicamente a altos níveis deste poluente. Além disso, observamos que estas alterações se estenderam aos frutos, culminando em diminuição da produtividade e modificação da qualidade dos mesmos (Figura 5). Os frutos de plantas ozonizadas apresentaram uma alteração em composição química, porém isso se refletiu negativamente sobre o potencial antioxidante dos mesmos. Portanto, podemos dizer que a exposição ao ozônio alterou a qualidade do fruto; porém somente testes *in vivo* definirão se essas alterações acarretaram em melhora ou piora no potencial terapêutico da pimenta.

Aparentemente, as folhas foram mais sensíveis ao O_3 do que os frutos, possivelmente porque este poluente altamente reativo entre na planta através de orifícios encontrados na superfície foliar (estômatos). As folhas apresentaram danos visíveis como clorose e ondulamento das bordas (Figura 4). Já os frutos de plantas ozonizadas, com exceção da quantidade diminuída, não apresentaram danos visíveis.

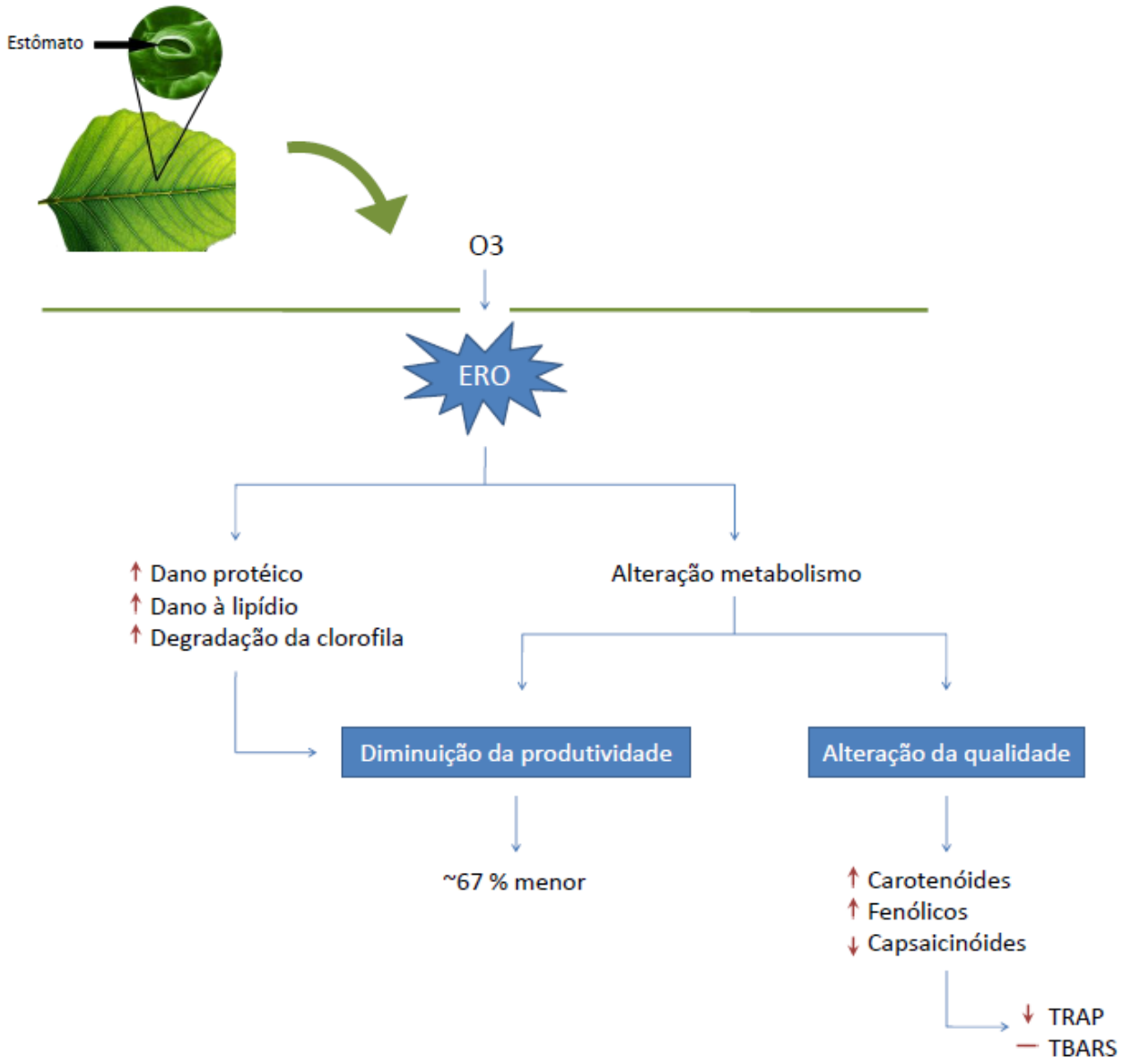

Figura 5: resumo esquemático sintetizando os resultados do trabalho.

6.2 Específicas

Foi observado no tecido foliar de plantas expostas ao O_3 um aumento na lipoperoxidação e carbonilação protéica, níveis de ERO aumentados, degradação de clorofila aumentada, conteúdo de fenólicos totais diminuídos, atividade enzimática alterada, conteúdo de antocianinas aumentado. Além disso, uma análise de correlação mostrou que os compostos fenólicos podem

estar relacionados positivamente com a prevenção ao dano lipídico causado pelo O_3.

Em relação aos frutos, nós observamos que a exposição ao O_3 diminuiu a produtividade dos frutos. Além disso, os frutos apresentaram níveis diminuídos de capsaicina, porém níveis aumentados de carotenóides e polifenóis totais, bem como de zeaxantina. Por fim, constatamos uma diminuição do potencial antioxidante dos frutos tratados.

7. ANEXO - RESULTADOS COMPLEMENTARES

7.1. Material e métodos

7.1.1. Condições de cultivo e modo de exposição

As condições de cultivo, bem como a maneira como foi conduzida a exposição são as mesmas descritas nos tópicos 2.1, 2.2 e 2.3 do artigo presente na seção de resultados desta dissertação. Brevemente, sementes de *Capsicum baccatum* L. var. *pendulum* accesso “Pimenta-dedo-de-moça” foram plantadas em substrato padronizado. Após a germinação estas plantas foram cultivadas durante 75 dias (início da floração). Estas plantas, então, foram transferidas para câmaras de topo aberto onde foi feita a exposição ao ozônio. Seis câmaras de topo aberto foram usadas para o experimento, sendo que três delas foram alimentadas com o ar ambiente (câmaras controles) e outras três com ar ambiente mais ozônio, o qual foi produzido por um ozonizador. Em cada câmara de topo aberto foram colocadas cinco plantas somando um total de 30 indivíduos (15 controles e 15 tratados). Para facilitar o entendimento da exposição ao O_3 a Figura 1 trás um diagrama esquemático de um sistema de cultivo de plantas em atmosferas enriquecidas com O_3. A exposição ocorreu durante todo o desenvolvimento do fruto (62 dias). Durante este período o ozônio foi monitorado, sua concentração variou de 125.9 a 251.8 µg/m^3 (média = 171.6 µg/m^3 ± 44.79) nas câmaras com acréscimo de ozônio e não pode ser detectado nas câmaras controles.

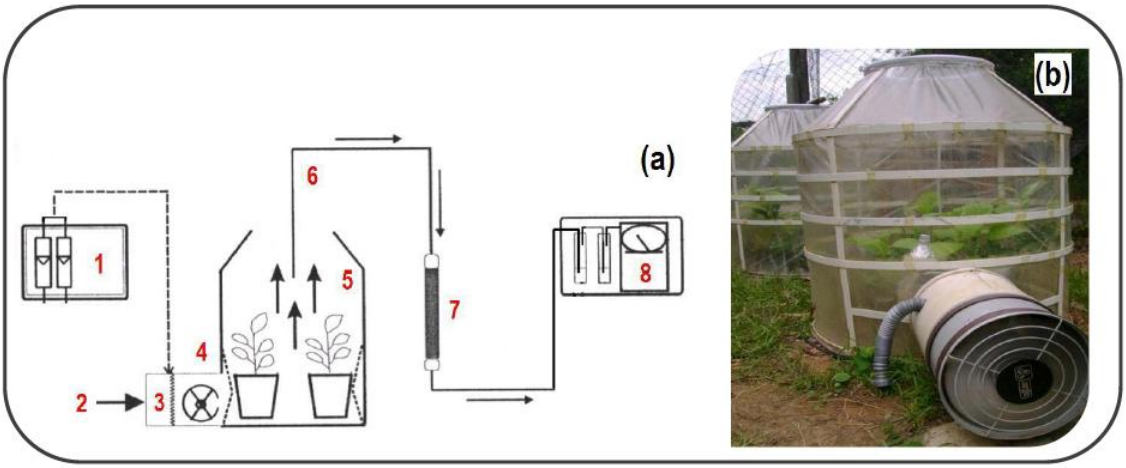

Figura 6: Diagrama esquemático de um sistema para cultivo de plantas em atmosferas enriquecidas com O_3. 1: Ozonizador, 2: Entrada de ar para a câmara de topo aberto, 3: Câmara de homogeneização, 4: Ventilador homogeneizador de ar, 5: Câmara de topo aberto, 6: Tubulação para amostra de ar para medição, 7: Filtro de umidade, 8: Amostrador atmosférico para O_3. b) Foto das câmaras de topo aberto utilizadas no experimento.

7.1.2. Coleta dos frutos e preparação dos extratos

Após 62 dias, frutos maduros (Figura 2) foram coletados, lavados, separados das sementes e estocados no freezer a - 20 ºC. Para a preparação dos extratos os frutos foram descongelados e cortados em pedaços menores com cerca de quatro cm^2, os quais foram submetidos à extração por decocção com solução hidroalcoólica de 40% (fruto:solvente, 1:10, w/v) durante 20 min. O extrato foi filtrado e em seguida o solvente foi eliminado em rota-evaporador. Os extratos secos foram armazenados em freezer -20 °C para experimentos posteriores.

7.1.3. Análises

Devido à hidrofilicidade do extrato, para a realização das análises todos os extratos foram solubilizados em água destilada na concentração de 100.000 µg/mL (solução estoque).

7.1.3.1. Quantificação de fenólicos totais

Os fenólicos totais foram determinados pelo método do Folin-Ciocalteu (Singleton e Rossi, 1965). As amostras foram misturadas com o reagente Folin-Ciocalteu 1M e carbonato de cálcio (35 % w/v). O conteúdo de fenólicos foi determinado colorimetricamente a 765 nm e expresso em µg de equivalentes de ácido gálico (EAG)/g de extrato.

7.1.3.2. Quantificação de carotenóides

Para a extração dos carotenóides alíquotas dos extratos já solubilizados em água foram misturadas com MTBE (3:1, v/v). Esta solução foi fortemente agitada e em seguida centrifugada a 10.000 g durante 10 minutos para a separação das fases. A fase superior foi coletada e filtrada através de uma membrana PTFE modificada para filtração de solventes orgânicos e aquosos com 0,45 µm da Millex para análise em HPLC e espectrofotômetro. Os carotenóides totais foram analisados espectrofotometricamente a 450 nm. O β-caroteno solubilizado em MTBE foi usado como padrão de referência para a realização da curva padrão. Os resultados foram expressos em µg de equivalentes de β-caroteno (EBC)/g de extrato.

A análise dos teores de carotenóides foi realizada por cromatografia líquida de alta eficiência (HPLC), empregando cromatógrafo líquido Agilent series 1100, Santa Clara, CA, USA, bomba quaternária e amostrador

automático. Os dados foram processados usando o software TotalChrom® Workstation. As análises foram realizadas em temperatura ambiente (24 ± 2 °C) usando como fase estacionária uma coluna YMC C_{30} 250 micrometro x 4.6 micrometro; tamanho de partícula 3 mM . A fase móvel foi constituída de água/metanol/MTBE a 5:90:5 e alterando para 0:95:5 depois de 12 minutos, 0:89:11 depois de 25 minutos, 0:75:25 depois de 40 minutos e 00:50:50 depois de 60 min, com um fluxo de 1 mL/minutes a 33 °C. Zanatta e Mercadante, (2007). A detecção foi realizada em 450 nm e os espectros de UV obtidos em 250-600 nm. As amostras foram injetadas na concentração de 300 mg/mL e o volume de injeção foi de 5 µL. A identificação dos carotenóides (luteína, β-criptoxantina, zeaxantina, α-caroteno e β-caroteno) foi baseada no tempo de retenção e co-injeção com padrões e respectivos espectros de ultravioleta. Todas as análises foram realizadas em triplicata.

A quantificação foi baseada na curva padrão para cada carotenóide. A curva padrão foi de 5 a 50 µg/mL para β-caroteno, 2 a 25 µg/mL para α-caroteno, 1 a 65 µg/mL para luteína, 4 a 100 µg/mL para criptoxantina e 1 a 40 µg/mL para zeaxantina. Os limites de detecção e de quantificação foram determinados como descrito previamente por Long e Winefordner, (1983). Os limites de detecção e de quantificação foram respectivamente de 6.5x10-2 e 10.9x10-2 mg/kg para β-caroteno; 6.9x10-3 e 1.2x10-2 mg/kg para luteína; 2.1x10-2 e 3.5x10-2 mg/kg para criptoxantina; 9.6x10-2 e 1.6x10-2 mg/kg para zeaxantina; e 2.0x10-2 e 3.3x10-2 mg/kg para α-caroteno.

7.1.3.3. Quantificação de capsaicinóides

A análise dos teores de capsaicina e dihidrocapsaicina foi realizada por cromatografia líquida de alta eficiência (HPLC), empregando cromatógrafo líquido PerkinElmer Series 200, equipado com detector de ultravioleta, bomba binária, forno de coluna e amostrador automático. Os dados foram processados usando o software TotalChrom® Workstation. As análises foram realizadas a temperatura de 40°C usando como fase estacionária uma coluna Brownlee® Choice C-18 (150 × 4,6 mm i.d.; 5 µm). A fase móvel foi constituída de um gradiente entre os solventes acetonitrila (A) e solução aquosa de ácido acético 1% (pH = 3) (B), nas seguintes condições: 1 - 13 minutos isocrático A-B (45:55 v/v); 13 - 16 minutos gradiente linear de A-B (45:55 v/v) para A-B (60:40); e 16 - 22 minutos isocrático A-B (60:40 v/v), com fluxo constante de 1 mL/min. Os extratos de *C. baccatum* foram injetados na concentração de 30 mg/mL, com volume de injeção foi de 20 µL, e detecção em 280 nm. A identificação da capsaicina e dihidrocapsaicina foi baseada no tempo de retenção e co-injeção com padrões. Todas as análises foram realizadas em triplicata.

7.1.3.4. Determinação do Potencial Antioxidante Total (TRAP) e da Reatividade Antioxidante Total (TAR)

O TRAP e o TAR foram mensurados e calculados como descrito previamente por Dresch et al. (2009) e Lissi et al. (1995). Brevemente, a solução de reação contendo AAPH (10 mM) e luminol (4 mM) em tampão glicina (100 mM, pH 8,6) foi incubada a 21 °C por duas horas. AAPH é uma fonte geradora de radical peroxil, o qual reage com luminol produzindo quimiolumiscência (CL). Após duas horas de incubação 20 µL de amostra (concentração final de 20 µg/mL), Trolox (conentração final de 1 µM) ou veículo

(água destilada). O perfil do TRAP foi obtido pela medida da quimioluminescência em um cintilador líquido. Os resultados foram transformados em porcentagem para calcular a área sob a curva. Uma menor área significa um maior potencial antioxidante total. O TAR, índice determinado pela medida do decaimento inicial da quimioluminescência, foi calculado pela razão entre I_0 e I, onde I_0 é a emissão inicial de quimioluminescência e o I é a medida da instantânea quimioluminescência após a adição das amostras ou Trolox.

7.1.3.5. Avaliação da inibição da lipoperoxidação pelo extrato (TBARS) *in vitro*

A mensuração das substâncias reativas ao ácido tiobarbitúrico (TBARS) é amplamente utilizada na literatura para avaliar a peroxidação lipídica (Hodges et al., 1999). Para avaliar o potencial do extrato de proteção contra o dano a lipídios a técnica foi adaptada, como descrita por (da Silva et al, 2007). Para isso, foi usado gema de ovo como uma fonte de lipídios. Brevemente, 1 mL da gema homogeneizada (1% peso/v) em 20 mM tampão fosfato (pH 7,4) foi misturado com os 0,1 mL de extrato em diferentes concentrações ou com controles (Trolox, ácido gálico ou veículo). Em seguida, foi adicionado 0,1 mL de uma solução de AAPH (120 mM) para induzir dano. Trolox (concentração final de 0,33 mM) e ácido gálico (concentração média de fenólicos presente nos extratos) foram usados como moléculas antioxidantes de referência (controle positivo) e o veículo (água) foi usado como controle negativo. A reação foi incubada durante 30 minutos a 37 °C. Após resfriamento as proteínas das amostras foram precipitadas com TCA (10 %) e centrifugadas a 10.000 g por

10 minutos. O sobrenadante foi misturado ao TBA (0,67 %) na proporção 1:1 e isso foi aquecido a 100 °C durante 30 minutos. Após o esfriamento as amostras foram lidas a 532 nm. Os resultados foram expressos em equivalentes de TMP/mL.

7.2. Resultados

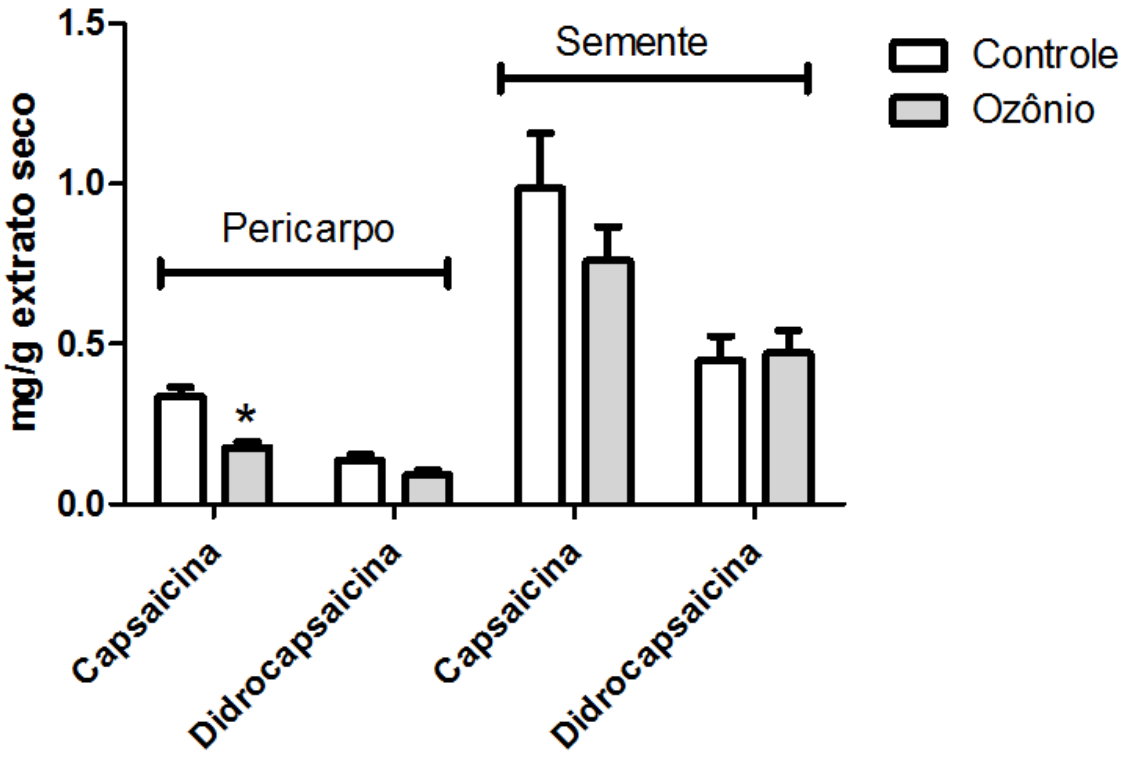

Figura 7: Concentração de capsaicina e didrocapsaicina em diferentes partes do fruto de plantas controles e expostas ao ozônio. Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$.

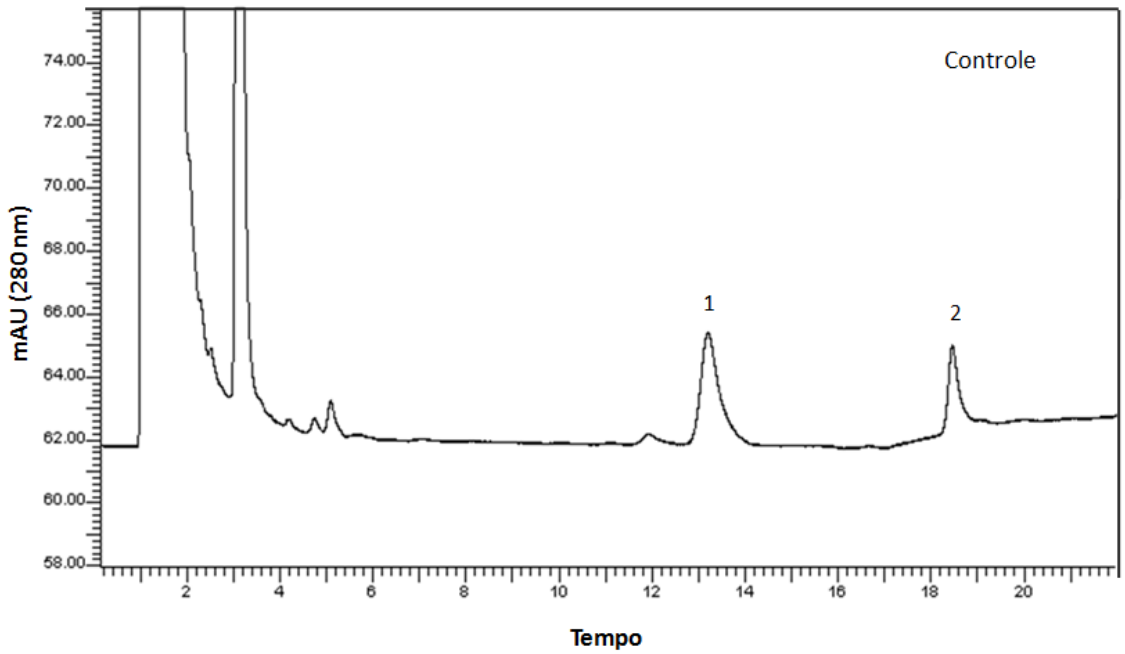

Figura 8: cromatograma de uma amostra controle mostrando os picos da capsaicina (1) e didrocapsaicina (2) detectados em HPLC a 280 nm.

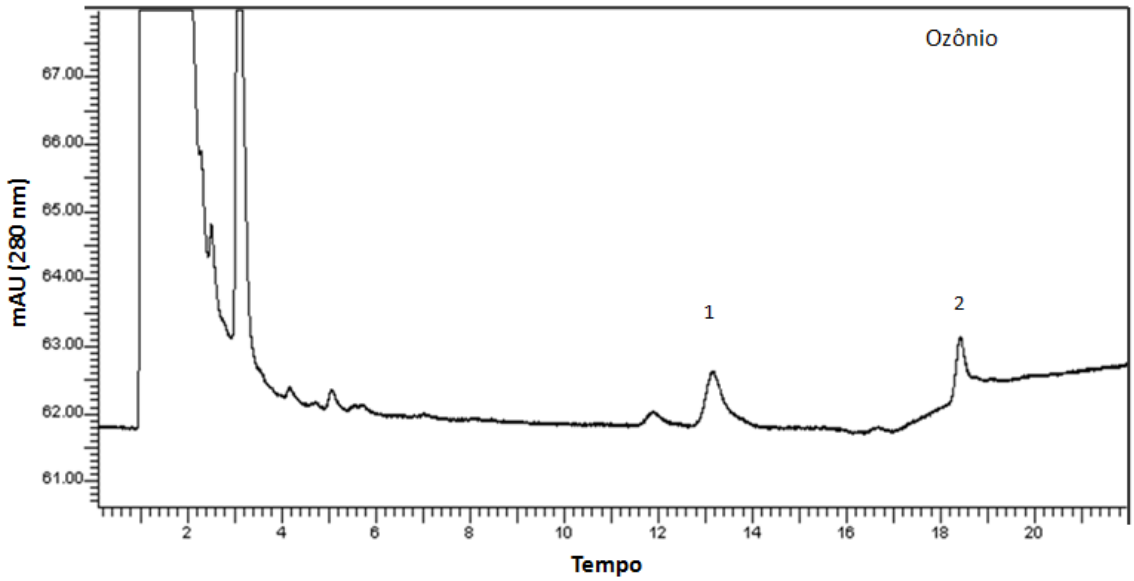

Figura 9: cromatograma de uma amostra tratada com ozônio mostrando os picos da capsaicina (1) e didrocapsaicina (2) detectados em HPLC a 280 nm.

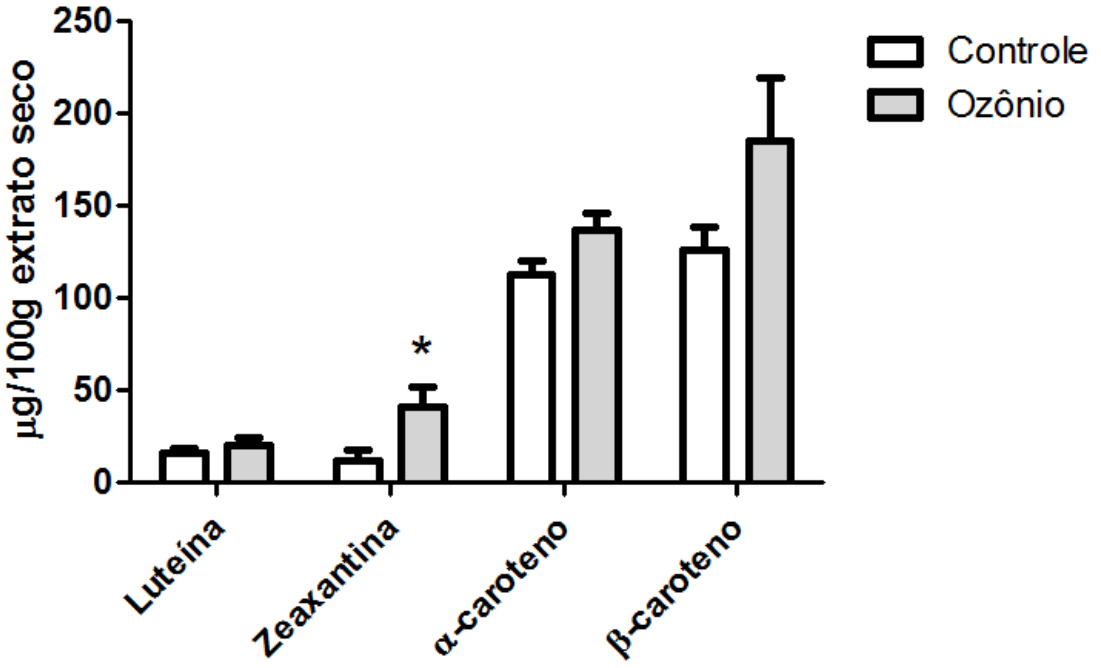

Figura 10: Concentração dos carotenóides (Luteína, Zeaxantina, α-caroteno e β-caroteno) nos frutos de plantas controles e expostas ao ozônio. Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$.

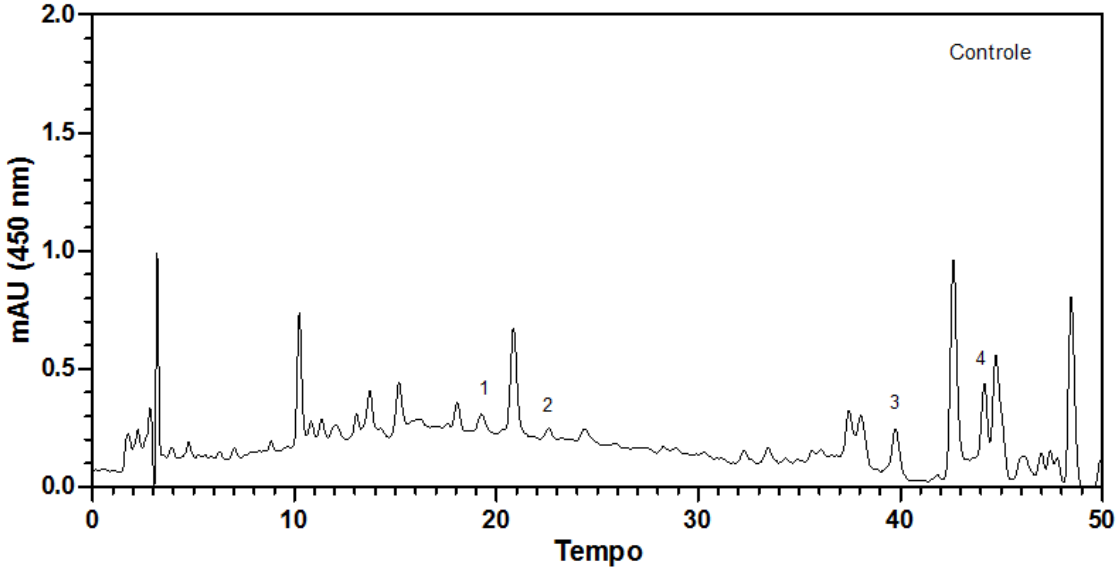

Figura 11: cromatograma de uma amostra controle mostrando os picos de luteína (1), zeaxantina (2), α-caroteno (3) e β-caroteno (4) detectados em HPLC a 450 nm.

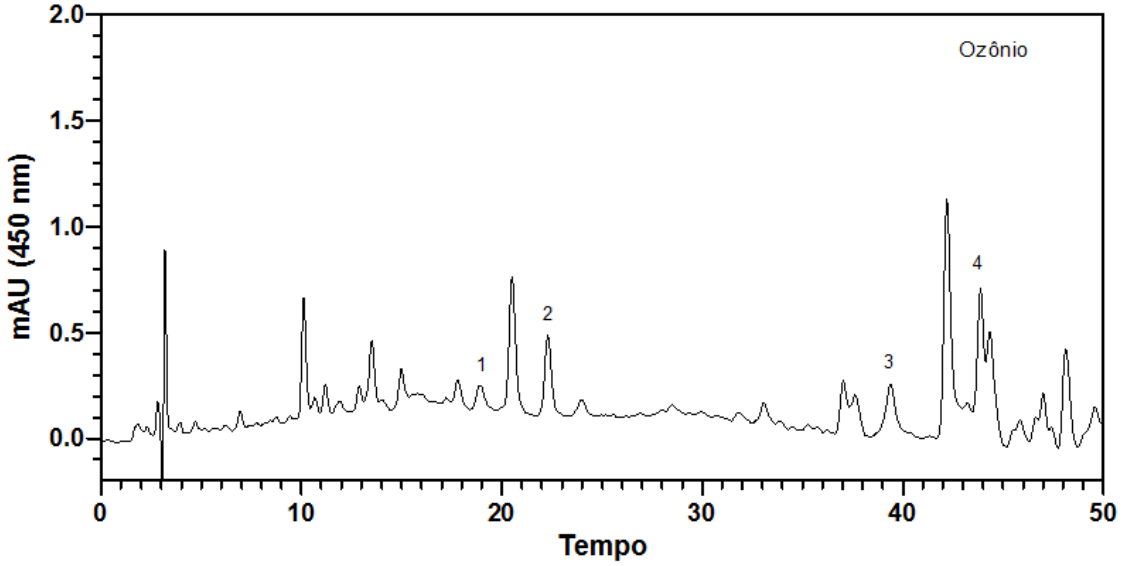

Figura 12: cromatograma de uma amostra tratadas com ozônio mostrando os picos de luteína (1), zeaxantina (2), α-caroteno (3) e β-caroteno (4) detectados em HPLC a 450 nm.

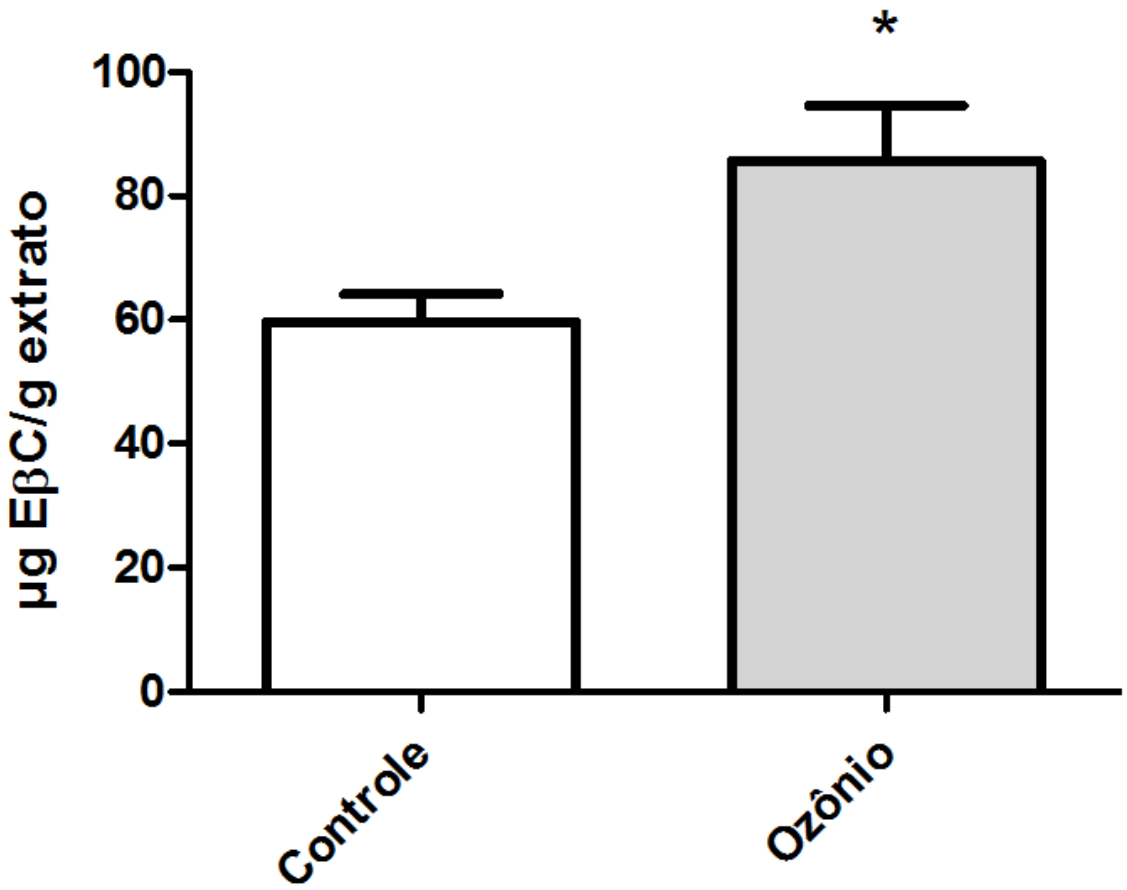

Figura 13: Concentração dos carotenóides totais nos frutos de plantas controles e expostas ao ozônio. Estes resultados foram obtidos em espectrofotômetro a 450 nm e foram expressos em µg de equivalente de β-

caroteno por grama de extrato seco (µg EβC/g extrato seco). Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$. Obeservação: β-caroteno foi usado como padrão para a construção da curva padrão.

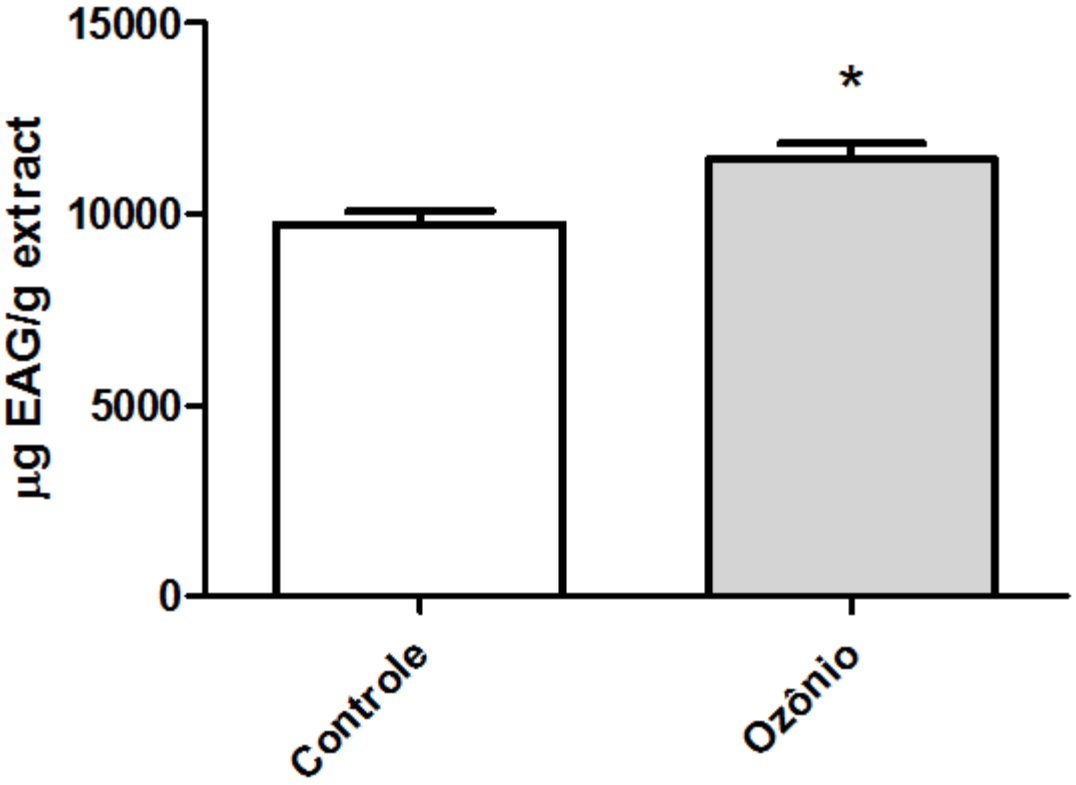

Figura 14: Concentração dos fenólicos totais nos frutos de plantas controles e expostas ao ozônio. Estes resultados foram obtidos em espectrofotômetro a 765 nm e foram expressos em µg de equivalente de ácido gálico por grama de extrato seco (µg EAG/g extrato seco). Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$. Obeservação: o ácido gálico foi usado como padrão para a construção da curva padrão.

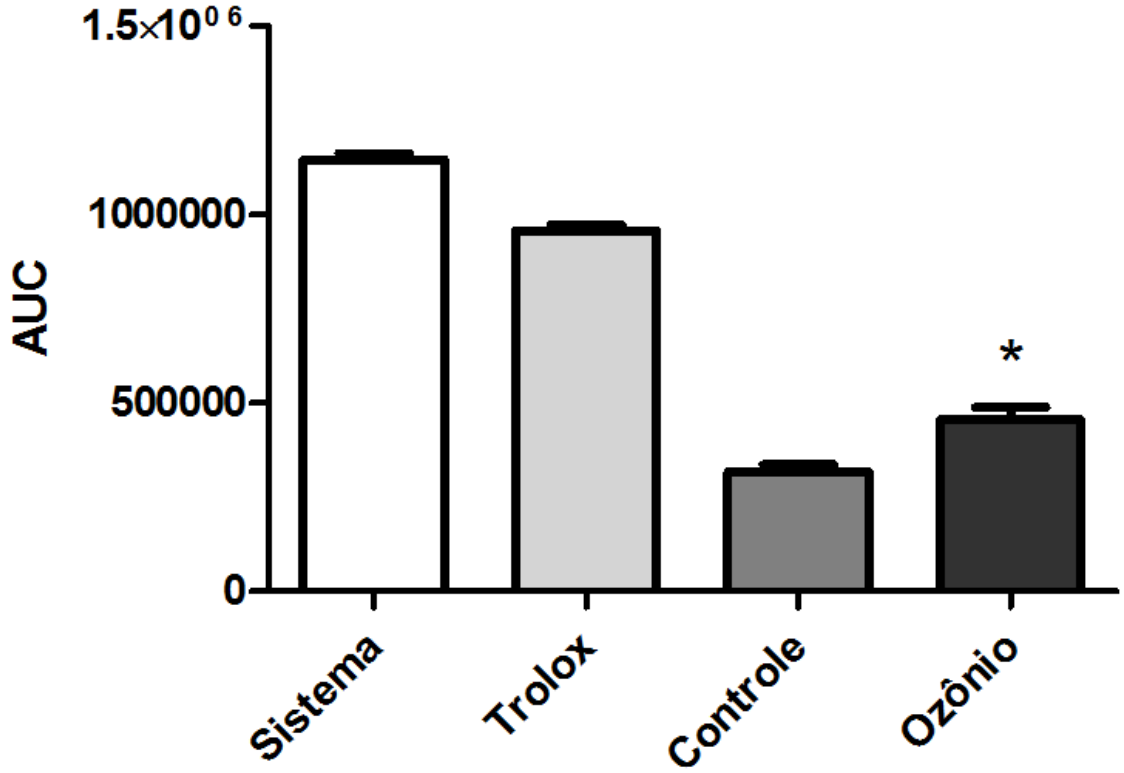

Figura 15: valores de área sob a curva (AUC) representando o potencial antioxidante, o qual é inversamente proporcional a área sob a curva. Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$. Obeservação: o Trolox foi usado como controle positivo da reação.

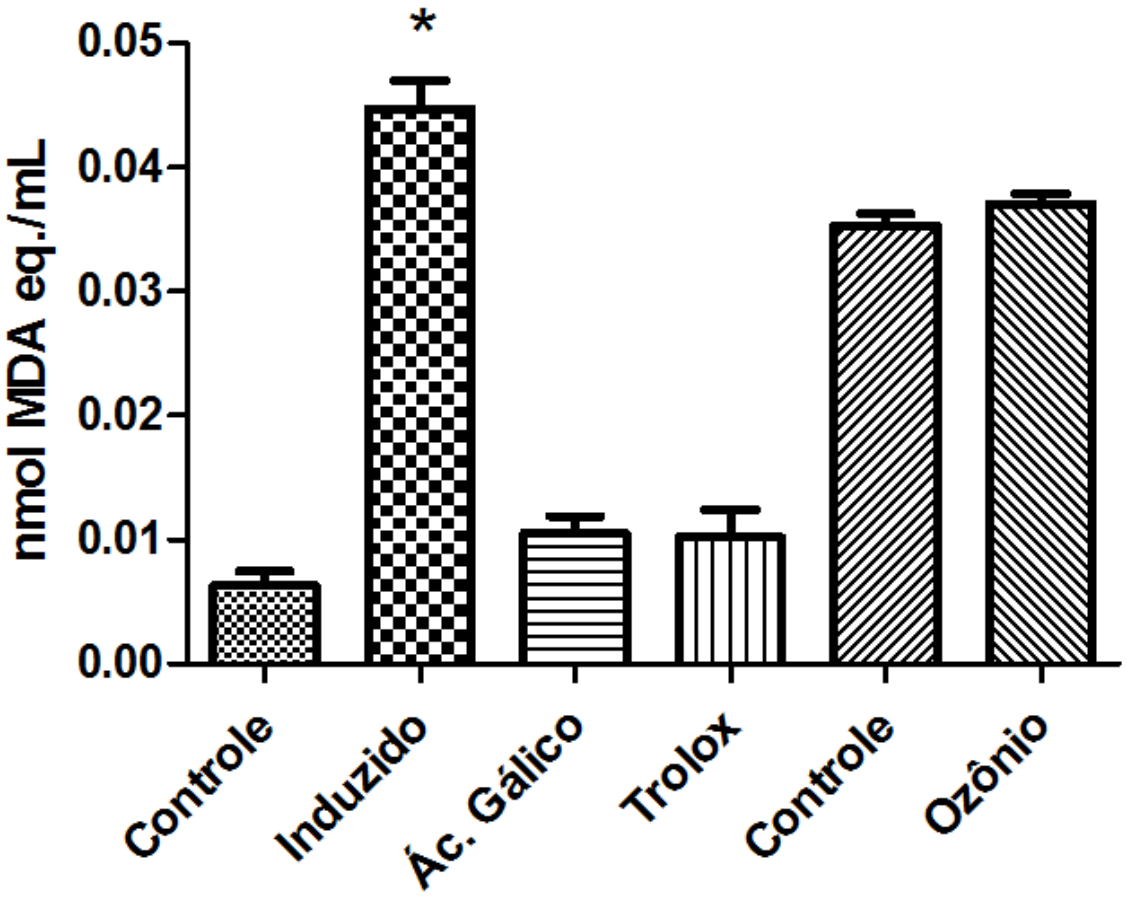

Figura 16: valores de inibição da lipoperoxidação mostrados em equivalentes de nmol de malondialdeído por mL (nmol MDA eq./mL), onde o induzido apresenta o máximo de lipoperoxidação. Dados mostrados como média ± erro padrão. Foi usado o teste-t de Student para avaliar as diferenças entre grupos controles e tratados, considerando como significativamente diferente $p<0,05$. Obeservação: o Trolox e o ácido gálico foram usados como controle positivo da reação.

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