UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

RESPOSTAS FISIOLÓGICAS E METABÓLICAS FRENTE A ESTRESSES EM *EUCALYPTUS GRANDIS X E. UROPHYLLA* E *ARAUCARIA ANGUSTIFOLIA*

JANIELI CRISTINA PEROTTI

PORTO ALEGRE, 2015.

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Tese submetida ao Programa de Pós-Graduação em Botânica da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do **Grau de Doutor**

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SE NÃO PUDER VOAR, CORRA

SE NÃO PUDER CORRER, ANDE

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MAS CONTINUE EM FRENTE DE QUALQUER JEITO!

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LISTA DE ABREVIATURAS

SuA : ácido sulfúrico, do inglês “sulfuric acid”

JA: ácido jasmônico, do inglês “jasmonic acid”

MeJA: metil jasmonato, do inglês “methyl jasmonate”

SA: ácido salicílico, do inglês “salicylic acid”

ET: ethrel

CEPA: 2-cloroetil-fosfônico, do inglês “chloro ethyl phosphonic acid”

NO: óxido nítrico, do inglês “nitric oxide”

SNP: nitroprussiato de sódio, do inglês “sodium nitroprusside”

SN: nitrato de prata,do inglês “silver nitrate”

CO_2: dióxido de carbono

A: taxa fotossintética

g_s: condutância estomática

E: taxa transpiratória

$A\backslash E$: eficiência transpiratória instantânea

WUE: eficiência do uso da água, do inglês “water use efficiency”

V_c: taxa de carboxilação da rubisco

J_{ETR}: Taxa máxima de transporte de elétrons

TPU: utilização da triose fosfato, do inglês “thriose-phosphate usage”

R_d: Respiração noturna foliar

Fv\Fm: relação entre fotossíntese variável e máxima – eficiência fotoquímica máxima do fotossistema II

Fv\Fo: razão entre fluorescência variável e inicial – performance das reações luminosas do fotossistema II

Fo\Fm: razão entre fluorescência inicial e máxima - rendimento quântico da dissipação

Pi_{abs}: índice de performance relativo a absorção

Pi_{total}: índice de performance total

BSA: albumina do soro bovino, do inglês “bovine serum albumine”

EC: elevada concentração de CO_2

ET: elevada temperatura

RGR: taxa de crescimento relativo, do inglês “relative growth rate”

NADH: nicotinamida adenina dinucleotídeo, do inglês “nicotinamide adenine dinucleotide”

RUBISCO: ribulose 1,5 bisfosfato carboxilase\oxigenase, do inglês “ribulose-1,5-bisphosphate carboxylase/oxygenase”

RuBP: ribulose 1,5 bisfosfato, do inglês “tibulose-1,5-bisphosphate”

RESUMO

O conhecimento sobre os mecanismos e a modulação de respostas de estresse em plantas arbóreas é necessário para a exploração sustentável destas importantes fontes de produtos de interesse industrial, também essenciais do ponto de vista ecológico, na regulação do clima e caracterização de diferentes biomas. Nesta tese, foram investigados dois exemplos de estresse em indivíduos jovens de espécies arbóreas, sendo um de uma gimnosperma frente a estresse biótico e outro de uma angiosperma submetida a estresse abiótico: controle da produção de resina por ferimento e moléculas sinalizadoras em *Araucaria angustifolia* (Bertol.) Kuntzee respostas fisiológicas de um híbrido comercial de eucalipto (*Eucalyptus grandis* W. Hill X *E. urophylla* S.T. Blake) à elevação de CO_2 e temperatura tendo como fonte de nitrogênio nitrato. No primeiro caso a hipótese testada foi de que a conífera basal *A. angustifolia* apresentaria controles de regulação distintos de coníferas mais avançadas, como *Pinus* spp. No segundo caso, a hipótese testada foi de que, sob temperatura ambiente moderada, haveria limitação no uso do excedente de CO_2 pelas plantas C_3, devido a dificuldades na assimilação de nitrogênio por falta de poder redutor, situação que seria parcialmente compensada por fotorrespiração sob alta temperatura sem restrição hídrica, devido à recuperação da disponibilidade de ácidos orgânicos, poder redutor e consequente assimilação do nitrato. No caso da produção de resina em araucária, a metodologia envolveu basicamente a remoção de acículas (ferimento) e a aplicação de moléculas estimuladoras e sinalizadoras de exsudação de resina conhecidas para *Pinus elliottii*, espécie mais derivada e de uso comercial, seguida do monitoramento do volume de resina produzido, bem como análise de sua composição por CG-MS. Já para o efeito da elevação de CO_2 e temperatura em eucalipto, foram avaliados tratamentos em câmara de crescimento Conviron incluindo condições controle (25 ºC, 379 ppm CO_2), elevado CO_2 (EC, 730 ppm CO_2, 25 ºC), elevada temperatura (ET, 30ºC, 379 ppm CO_2) e a combinação destes(EC+ ET, 30ºC, 730 ppm CO_2). Monitorou-se parâmetros de crescimento, parâmetros fotossintéticos e de fluorescência da clorofila, bem como o teor de carboidratos, fenólicos, antocianinas, proteínas solúveis e a atividade de nitrato redutase. No que tange à produção de resina em araucária, a hipótese foi refutada, tendo sido verificado que moléculas sinalizadoras típicas para produção de resina em *Pinus* também foram ativas em araucária, destacando-se etileno, jasmonato e ácido salicílico. Além disso, verificou-se pela primeira vez modulação de produção de resina por nitroprussiato de sódio, um precursor de óxido nítrico. No caso das respostas de eucalipto a simulação de cenários climáticos futuros com fonte oxidada de nitrogênio, os principais resultados foram o melhor desempenho fotossintético e o maior crescimento em plantas sob ET ou EC+ET, parcialmente apoiando a hipótese de que a falta de poder redutor limita o aproveitamento de CO_2 por plantas C3 sob condições não condutivas à fotorrespiração, indicando, portanto, a possibilidade de uma resposta compensatória da alta temperatura em relação às dificuldades de assimilação de nitrato, particularmente sob alto CO_2 em cenários climáticos futuros.

Palavras-chave: estresse, resina, ferimento, sinalização, terpenos, CO_2, temperatura

ABSTRACT

Understanding the mechanisms and the modulation of stress responses in trees is needed for sustainably exploring these important sources of products of commercial interest, which are also essential from an ecological viewpoint, in climate regulation and by characterizing different biomes.In this thesis, two examples of stresses on tree plantlets were investigated, one involving a gymnosperm under biotic stress and the other an angiosperm facing abiotic stress: control of resin production upon wounding and by signaling molecules in *Araucaria angustifolia* (Bertol.) Kuntze, and physiological responses of a commercial hybrid of eucalypt (*Eucalyptus grandis* W. Hill X *E. urophylla* S.T. Blake) having nitrate as sole N source to high CO_2 and heat. In the first case, the hypothesis tested was that the basal conifer *A. angustifolia* would show regulatory controls diverse from those of more advanced conifers, such as *Pinus* spp. In the second case, it was tested the hypothesis that under moderate temperature there would be a limitation in the use of excess CO_2 by C_3 plants due to difficulties in assimilating nitrogen as a result of low reducing power, a situation that could be partly compensated by photorespiration under high temperature and no water restriction, because of a recovery of organic acid availability, reducing power, and consequent nitrate assimilation. In the case of araucaria resin, the methodology consisted in removing needles (wounding) and applying stimulatory and/or signaling molecules of resin exudation know to operate in *Pinus elliottii*, a more derived species used commercially, followed by monitoring the volume of resin yielded, as well as its composition by GC-MS. For the study of the effect of CO_2 elevation and high temperature in eucalypt, the treatments evaluated in a Conviron growth chamber were control conditions (25ºC, 379 ppm CO_2) elevated CO_2 (EC, 730 ppm CO_2, 25ºC), elevated temperature (ET, 30ºC, 379 ppm CO_2), and combination of these last two conditions (EC+ ET, 30ºC, 730 ppm CO_2). Several indicators were monitored, including: growth and photosynthetic parameters, chlorophyll fluorescence, as well as the content of carbohydrates, phenolics, anthocyanins, soluble proteins, and nitrate reductase activity. With regards to the production of resin in araucaria, the hypothesis was rejected, since signaling molecules, typical stimulators of resin in *Pinus*, were also active in araucaria, particularly ethylene, jasmonate and salicylic acid. Besides, for the first time, it was shown that resin production could be modulated by sodium nitroprussiate, a precursor of nitric oxide. As far as eucalypt responses to simulations of future climate scenarios under a nitrate-only source of nitrogen, the main results were the better photosynthetic performance and growth of plants under ET and EC+ET, partially supporting the hypothesis that the lack of reducing power limits the benefit and effective use of CO_2 by C_3 plants under conditions not leading to photorespiration. Thereby, this indicates the possibility of a compensatory response of high temperature to overcome the difficulties of nitrate assimilation, particularly under high CO_2in future climate scenarios.

Keywords: stress, resin, wounding, signaling, terpenes, CO_2, temperature

INTRODUÇÃO

INTRODUÇÃO

O Brasil apresenta uma das floras mais diversas e exuberantes do planeta. Dentre as espécies vegetais, cerca de 7.880 são arbóreas. As Angiospermas são o grupo mais diverso dentre todas as plantas. Acredita-se que há entre 30.000 e 35.000 espécies de Angiospermas em todo território brasileiro. As Gimnospermas são pouco representadas, com apenas 14 espécies identificadas (Shepherd, 2005).

Várias espécies arbóreas destacam-se pela qualidade da sua madeira (celulose, fibras, polpa e biomassa para produção de energia), uso medicinal e industrial, produção de mel e utilização ornamental e paisagística. Espécies nativas possuem também grande relevância ecológica através do reflorestamento e recomposição de áreas ambientalmente degradadas, fonte de alimento e refúgio para a fauna silvestre, habitat para epífitas como bromélias, pteridófitas e orquídeas, sequestro de carbono atmosférico, fixação de nitrogênio e ciclagem de matéria orgânica no solo.

Durante muitos anos as florestas nativas foram as principais fontes de energia oriunda da biomassa florestal. Em meados do século XIX, a indústria madeireira começou a se desenvolver no Sul do Brasil, devido à grande disponibilidade de araucária, matéria prima de apreciável qualidade. A partir da década de 60, com a redução da oferta de araucária oriunda do Sul e do processo de ocupação e integração da região Amazônica, ocorreu uma gradativa transferência do parque industrial madeireiro para o norte do país (Tonello *et al*. 2006).

Posteriormente, as florestas de eucalipto passaram a ter participação fundamental e de destaque na oferta interna de energia da biomassa. O Brasil passou a ter uma ampla área de plantações florestais, especialmente de eucalipto, uma das maiores do mundo, com mais de 5 milhões de hectares (Colodette *et al*. 2014). O uso de florestas comerciais plantadas é de grande importância para a redução de pressão sobre florestas nativas e sustentabilidade da indústria de derivados florestais.

A biomassa florestal possui características tais que permitem a sua utilização como fonte alternativa de energia, seja pela queima da madeira, como carvão, aproveitamento de resíduos da exploração e aproveitamento de óleos essenciais, alcatrão e ácidos pirolenhosos (Couto *et al*. 2000). O acúmulo da biomassa é influenciado por todos os fatores que afetam a fotossíntese e a respiração. A produtividade de um ecossistema está relacionada diretamente com o consumo e

com a disponibilidade de CO_2 no ambiente, pois esta é uma molécula central no processo de acúmulo de biomassa das plantas, assim como com a água, a radiação solar e os nutrientes minerais (Caldeira, 2003; Urbano, 2007).

As espécies vegetais apresentam um papel fundamental no ciclo do carbono, contribuindo na diminuição do CO_2 atmosférico ao servir como 'sumidouro' de carbono a partir da fotossíntese e incorporação em moléculas de meia vida mais estável, como celulose e lignina. Desta forma, as florestas contribuem para a estabilidade ambiental, com a mitigação das temperaturas extremas, participando da regulação das precipitações, prevenindo a erosão e a deterioração do solo (Miranda, 2008; Silveira, 2008).

Segundo dados do IPCC (2014), as concentrações de CO_2 atmosférico aumentaram de 280 para 390μmol mol^{-1} desde 1800 e acredita-se que esses valores possam atingir de 530 a 970 μmol mol^{-1} até o final do século, o que resultaria em um aumento na temperatura de 0,3 a 1.7°C até 2.6 a 4,8°C na escala global. Desta forma, estima-se que as espécies arbóreas apresentem um papel fundamental, pois podem contribuir para reduzir a concentração de CO_2 atmosférico através da fotossíntese, atingindo assim, uma maior taxa de crescimento. No entanto, o potencial real para esta mitigação permanece incerto. Os efeitos de cenários climáticos futuros no crescimento, metabolismo primário e secundário de espécies arbóreas são pouco conhecidos, sobretudo quando se consideram as complexas relações envolvidas, incluindo assimilação de nitrogênio e fotorrespiração em combinações de alta temperatura e alto CO_2.

Quando as plantas sofrem algum tipo de estresse, biótico ou abiótico, elas respondem produzindo metabólitos secundários com funções de defesa, muitas vezes contra estresse oxidativo associado a estas condições (Matsura *et al*, 2014). A produção destes metabólitos ocorre mediada por várias moléculas sinalizadoras fitormonais, tais como ácido jasmônico, ácido salicílico, etileno, ácido abscísico e poliaminas. Estas moléculas sinalizadoras apresentam um papel crucial na adaptação das plantas ao ambiente e a condições estressantes, conferindo vantagens estratégicas em condições adversas pelo acúmulo de metabolitos de defesa (Nascimento e Fett-Neto, 2010).

Os produtos do metabolismo secundário podem ser divididos em três grandes grupos segundo a sua biossíntese: compostos fenólicos, alcalóides e terpenóides. Os compostos fenólicos estão presentes em todos os órgãos das plantas. Mais de 8000 compostos já foram caracterizados, compreendendo estruturas diversas que variam de moléculas simples, como os

ácidos fenólicos, a compostos cujas moléculas são altamente ramificadas, como os taninos (Quideau *et al*. 2011). Já os alcalóides são caracterizados pela presença de um átomo de nitrogênio em sua estrutura básica, sendo geralmente oriundos de aminoácidos. Constituem uma classe numerosa, encontrados em bactérias, fungos, animais e plantas, a qual é dividida em subclasses por apresentar vias de síntese não relacionadas evolutivamente (Cushnie *et al.* 2014).Os terpenóides, também conhecidos como terpenos ou isoprenóides, constituem uma classe de produtos naturais de plantas com a maior variedade estrutural e funcional, podendo apresentar funções tanto no metabolismo primário, como no secundário (Croteau *et al*. 2000).

Terpenoides são derivados de uma molécula precursora com cinco carbonos, o isopentenil difosfato (IPP). Os terpenoides atuam nas plantas em diferentes funções características, como por exemplo, na atração de polinizadores e dispersores de sementes (óleos essenciais e carotenóides), sinalizadores e defesa contra patógenos (compostos antibióticos e fitoalexinas), defesa contra herbivoria (repelentes), interações químicas entre as plantas (alelopatia), atuando como reguladores de crescimento (giberelinas, ácido abscísico) e como componentes estruturais da membrana (fitosterois). Alguns terpenos são farmacologicamente importantes, como o taxol (importante agente antitumoral), e outros são extensivamente utilizados na indústria química de flavorizantes e fragrâncias (Bohlmann e Keeling, 2008).

Muitas espécies de coníferas produzem resinas à base de terpenoides de grande importância industrial e que atuam na resposta de defesa da planta contra o ataque de herbívoros e patógenos. Esta resina, presente principalmente nas famílias Pinaceae e Araucarieceae, consiste de uma complexa mistura de mono (C_{10}), sesquiterpenos (C_{15}) voláteis e diterpenos (C_{20}) não voláteis (Langenheim, 2003; Rodrigues-Corrêa e Fett-Neto, 2013). Embora o conhecimento sobre os mecanismos envolvidos na produção de resina de *Pinus* seja relativamente detalhado, pouco se sabe sobre a regulação deste processo em *Araucaria* (Fett-Neto e Rodrigues-Corrêa, 2012).

Dentro deste contexto, visando contribuir para a melhor compreensão das respostas de espécies arbóreas a estresses ambientais, o presente estudo abordou dois exemplos distintos, englobando uma gimnosperma e uma angiosperma e estresses bióticos e abióticos, respectivamente, quanto a sua natureza. No primeiro capítulo "Control of resin production in *Araucaria angustifolia*, an ancient South American conifer" foi investigada a hipótese de que as coníferas ancestrais, de crescimento lento, com produção de resina apenas na casca, como é o

caso da espécie *A. angustifólia,* apresentariam diferenças significativas na regulação da produção de resina induzida por ferimento comparada a coníferas mais derivadas, de rápido crescimento e que produzem resina abundante, tanto na casca quanto no cerne, tais como *Pinus elliottii*. No segundo capítulo "Growth and metabolic responses of a commercial eucalypt hybrid exposed to high temperature and high CO_2 under nitrate supply" testou-se a hipótese de que, em plantas C_3, especialmente arbóreas, o aumento da fotorrespiração, induzido pela alta temperatura, poderia compensar a limitação na assimilação de nitrogênio na presença de alto CO_2 ('aclimatação ao carbono'), que é prejudicada quanto à redução de nitrato pela falta de ácidos orgânicos e poder redutor.

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CONTEÚDOS ABORDADOS

O **Capítulo I** apresenta um manuscrito publicado no periódico *Plant Biology*. Este capítulo abordao efeito de alguns estimuladores químicos (ethrel, ácido salicílico, ácido jasmônico, ácido sulfúrico e nitroprussiato de sódio) na produção e composição da resina de *A. angustifolia*.

O **Capítulo II** apresenta um manuscrito a ser submetido ao periódico *Plant Biology*. Neste estudo, nós simulamos um futuro cenário climático com altas concentrações de CO_2 atmosférico e temperatura elevada, e verificamos seus efeitos isolados e combinados no crescimento, trocas gasosas, fluorescência da clorofila a e na concentração de alguns metabólitos.

CAPÍTULO I

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RESEARCH PAPER

Control of resin production in *Araucaria angustifolia*, an ancient South American conifer

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ABSTRACT

Araucaria angustifolia **is an ancient slow-growing conifer that characterises parts of the Southern Atlantic Forest biome, currently listed as a critically endangered species. The species also produces bark resin, although the factors controlling its resinosis are largely unknown. To better understand this defence-related process, we examined the resin exudation response of *A. angustifolia* upon treatment with well-known chemical stimulators used in fast-growing conifers producing both bark and wood resin, such as *Pinus elliottii*. The initial hypothesis was that *A. angustifolia* would display significant differences in the regulation of resinosis. The effect of Ethrel® (ET – ethylene precursor), salicylic acid (SA), jasmonic acid (JA), sulphuric acid (SuA) and sodium nitroprusside (SNP – nitric oxide donor) on resin yield and composition in young plants of *A. angustifolia* was examined. In at least one of the concentrations tested, and frequently in more than one, an aqueous glycerol solution applied on fresh wound sites of the stem with one or more of the adjuvants examined promoted an increase in resin yield, as well as monoterpene concentration (α-pinene, β-pinene, camphene and limonene). Higher yields and longer exudation periods were observed with JA and ET, another feature shared with *Pinus* resinosis. The results suggest that resinosis control is similar in *Araucaria* and *Pinus*. In addition, *A. angustifolia* resin may be a relevant source of valuable terpene chemicals, whose production may be increased by using stimulating pastes containing the identified adjuvants.**

INTRODUCTION

Many conifer species produce terpenoid-based resins that have long been studied for their industrial importance and role in defence against attack by herbivores and pathogens. The two most important resin-producing families of conifers are Pinaceae and Araucariaceae (Langenheim 1996). The viscous resin secretion is generally composed of a complex mixture of terpenoids, consisting of roughly equal parts of volatile mono- (C_{10}) and sesquiterpene (C_{15}; turpentine) fractions and non-volatile diterpenic (C_{20}; rosin) components (Rodrigues-Corrêa *et al.* 2013). Terpenes act in a complex and multilayered defence response, providing toxicity against bark beetles and fungi, bark wound sealing, disruption of insect development and attraction of herbivore predators (Phillips & Croteau 1999).

Most conifers rely on some combination of preformed and inducible resin defences (Trapp & Croteau 2001; Zulak & Bohlmann 2010). Resin defences are controlled by environmental and genetic factors to various extents, depending on species (Roberds *et al.* 2003; Sampedro *et al.* 2010; Moreira *et al.* 2013). Resin traits have been reported as highly variable, having moderate heritability, indicating that breeding efforts towards super-resinous forests are promising (Tadasse *et al.* 2001; Roberds *et al.* 2003). Several chemicals are known as stimulants of resin production. Commercial extraction of resin from pine trees uses periodic bark streaking and application of resin stimulant pastes to the wound.

Resin-stimulant paste based on sulphuric acid (SuA) is widely used for the commercial production of pine resin. Current stimulant pastes usually have two chemically active components, SuA to magnify the wounding and an ethylene precursor (2-chloroethylphosphonic acid, CEPA or Ethrel® – ET) to stimulate resin flow (Rodrigues *et al.* 2011; Rodrigues-Corrêa & Fett-Neto 2013). Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJa), are among the most widely used chemical elicitors of plant secondary metabolism. It has been shown that the exogenous application of MeJa or herbivore attack induce chemical and anatomical defence responses in conifers, such as the formation of traumatic resin ducts and resin accumulation in stems, along with increased biosynthesis of terpenes and phenolics (Franceschi *et al.* 2002; Martin *et al.* 2002; Heijari *et al.* 2005; Zeneli *et al.* 2006; Moreira *et al.* 2008; Gould *et al.* 2009). JA commercial use, however, is limited by its high cost.

The effects of exogenous salicylic acid (SA) on conifer terpene production have also been studied. In *Pinus elliottii*, application of 10 $mol \cdot m^{-3}$ of SA induced resin production in wound panels, but in *Pseudotsuga menziesii* and *Sequoiadendron giganteum* it had no apparent effect on resin accumulation (Hudgins & Franceschi 2004; Rodrigues & Fett-Neto 2009). Nitric oxide (NO) has also emerged as an

important endogenous signalling molecule in plants, mediating several developmental and physiological processes (Yu *et al.* 2014). SA has been shown to induce NO production (Zottini *et al.* 2007), making NO a likely candidate for modulating resinosis.

Monoterpenes are abundantly synthesised by Araucariaceae. Compounds such as pinene, camphene and limonene are often found in Araucariaceae species (Wang *et al.* 1997). Sesquiterpenes of different classes have been reported in *Araucaria* spp., including cadinanes, caryophyllanes and humulanes (Otto & Wilde 2001). The resin of *Araucaria columnaris* contains sesquiterpene hydrocarbons and oxygenated sesquiterpenes, being rich in aromadendrene and bicyclogermacrene (Lebouvier *et al.* 2013). Diterpenes of three main chemical skeleton types, abietane, pimarane and labdane are present in Araucariaceae (Langenheim 2003). Terpenes present in gymnosperm resin find a number of applications in the chemical, pharmaceutical, food and biofuel industries (Bohlmann & Keeling 2008; Rodrigues-Corrêa *et al.* 2013).

Araucaria angustifolia (Bertol.) Kuntze (Brazilian pine) is the only representative of the Araucariaceae in Brazil. This tree characterises the Mixed Ombrophile Forest, one of the phytophysiognomies of the Atlantic Forest biome (Rambo 2000; Kershaw & Wagstaff 2001), and its seeds constitute a relevant source of food for local fauna (Reitz & Klein 1966). *A. angustifolia* original area in the early twentieth century has fallen to just 2–4% due to logging, pushing the species to the status of critically endangered (Guerra *et al.* 2002; IUCN 2012). In this context, effective defence traits, including those based on bark resin, are potentially relevant for survival of this threatened conifer species in its natural environment. *A. angustifolia* lacks wood resin canals or resinous axial tracheids that are often found in other members of Araucariaceae (Esteban *et al.* 2004). *A. angustifolia* resin is produced in the bark, having a reddish colour in mature plants. This product was explored in the 1960s and early 1970s for the production of turpentine, varnishes and fuel (FAO 1986, 1995; Farjon 2010). Leaves of *A. angustifolia* also have resin canals located among vascular bundles, as well as below the central vascular bundle along the leaf axis (Mastroberti & Mariath 2003).

Little is known about the role of chemical elicitors on the production of resin in *Araucaria*, in contrast to the well-known participation of various elicitors in resinosis of more recently diverged conifers, such as species of *Pinus* (Rodrigues-Corrêa *et al.* 2013). *Araucaria araucana* and *A. heterophylla* were shown to activate and induce new axial phloem resin ducts after MeJa treatment (Hudgins *et al.* 2004). The present study provides the first identification of factors capable of regulating *A. angustifolia* resinosis. Our hypothesis was that a slow-growing and bark resin-producing conifer, such as *A. angustifolia*, would have different controls of resin exudation compared to those known to operate in fast-growing conifers with both bark and wood resin production, such as *P. elliottii*. As experimental strategy, it was examined whether the species is responsive to chemical signals known to induce resin production in distantly-related, more advanced conifers. Therefore, the effect of ET (an ethylene precursor), SA, JA, SuA and sodium nitroprusside (SNP – an NO donor) on resin yield, composition and duration of exudation in young plants of *A. angustifolia* was evaluated.

MATERIAL AND METHODS

Plant material and growth conditions

Three- to 4-year-old plants of *A. angustifolia* were used in the experiments. Plants originated from mechanically scarified seeds to ensure homogeneous germination. Seeds were harvested from approximately 40 individuals growing in a single natural stand, mixed randomly for homogeneous distribution of genetic material among treatments within each experiment. The plants were grown in soil and vermiculite (1:1 v/v) in 2l plastic pots and maintained on benches close to glass windows, thus receiving natural daylight, at a temperature of 27 ± 5 °C. The pots were watered periodically and once a month received 0.5× MS (Murashige & Skoog 1962) basal medium (macro- and micronutrients only).

Experimental design

To study the production of resin, the pots containing the plants were transferred to a growth chamber with a temperature of 25 ± 2 °C and photoperiod 16-h light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by white fluorescent lamps. In all of the assays, which were in a totally randomised layout, five plants were used for each chemical treatment tested, and the experiment was independently repeated twice, using a different set of plants. In each plant, five leaves of the basal region of the stem were removed and 15 µl of the treatment solution applied on the leaf scar. Twenty-four hours after application of the chemical stimulant, the first exuded resin was collected with a spatula and weighed on an analytical balance. Immediately after resin mass determination, all samples were frozen and stored in an ultra-freezer (−80 °C). Plants continued to be monitored for a total of 3 weeks and any new resin exudation was harvested and added to the respective replicate and treatment to obtain the total resin yield.

Chemical stimulant treatments

Three SuA concentrations were tested: 1%, 5% and 10% (v/v) diluted in water + glycerol (10:1 v/v). The aqueous glycerol solution provided the needed viscosity to keep the resin stimulants being tested on the wound site. A control treatment with aqueous glycerol only was included.

After determining the optimal concentration of SuA for wounding and resin production, acid in this concentration was applied in three regions of the stem (apical, middle and basal) to determine the location yielding most resin. Once the most responsive stem portion was identified, the effect of other resin elicitors was tested on the same portion (apical). Three concentrations of SA (1, 10, 50 and 100 $mol \cdot m^{-3}$) and ET (50, 100 150 $mol \cdot m^{-3}$) were evaluated, as well as two of JA (50 and 100 $mol \cdot m^{-3}$) and SNP (50 and 100 $mol \cdot m^{-3}$). The solution preparation and method of application of the treatments were performed in the same way as previous treatments. The first resin collection was performed 24 h after application of stimulating solution. The plants continued to be observed for the occurrence of new exudation for 3 weeks. Subsequent collections were made weekly until resin exudation ceased completely. Again, control treatments consisting of aqueous glycerol alone were included in each assay.

Once the optimal concentration of each treatment for producing resin was established, the performance of each chemical stimulant was evaluated alone or combined with SuA: control (aqueous glycerol only), SuA, SA + SuA, SNP + SuA, JA + SuA and ET + SuA. Since jasmonate-induced defence response is mediated by ethylene, the effect of silver nitrate (SN), a known inhibitor of ethylene action, was tested on resin production, as well as the performance of JA and ET combined. The treatments were: control (aqueous glycerol only), SN (20 mol·m^{-3}), ET (150 mol·m^{-3}), ET + SN, JA (100 mol·m^{-3}), JA + SN and JA + ET. An outline of the experiments described in this report and the rationale of the treatments tested is provided in Table 1.

Extraction and analysis of monoterpenes

Analyses of monoterpenes were carried out using at least 80 mg resin per replicate. To obtain this amount of resin per replicate, the exuded resin of approximately 5–10 individuals was harvested (and pooled if needed) until the necessary amount of resin for adequate extraction and accurate component detection was reached. Because of the lower yields, higher numbers of individuals were included in the control treatment so that sufficient exuded material was obtained for the chemical analyses. In all of the treatments, biological triplicates of at least 80 mg resin each (derived from pooled exudates of 5–10 individuals; *i.e.*, a total of 15–30 plants per treatment, depending on individual yield) were extracted and analysed. For extraction, about 80 mg resin were transferred to a vial, and 3 ml diethyl ether added. Then, samples were kept in an ultrasonic bath at room temperature for 20 min. Samples were cleaned by passing through solid-phase extraction Extract-clean silica columns (Altech Inc., Columbia, MD, USA; 500 mg/8.0 ml). The clear solution obtained was then directly injected into a gas chromatograph (model GC-2010) with an auto-injector (Autosampler AOC-20i) connected to a mass spectrometer (Model GCMS-QP2010S). Splitless injections (1 µl) were made with an injector at a temperature of 220 °C. The column used was a Restek Rtx 5MS (30 m × 0.25 mm × 0.25 µm; Restek Corp., Bellefonte, PA, USA) with 95% polydimethylsiloxane: 5% phenyl, and temperature programmed to start at 40 °C (2 min hold) and increase at a rate of 5 °C·min^{-1} until 200 °C (1 min hold), followed by an additional ramp of 10 °C·min^{-1} up to 300 °C (5 min hold). The carrier gas, helium, was used at a constant flow of 1.02 ml·min^{-1} and linear velocity of 36.5 cm·s^{-1}.

For quantification of α-pinene, β-pinene, camphene and limonene in resin, standard curves were generated using authentic standard monoterpenes (Sigma-Aldrich, St. Louis, MO, USA); regression coefficients were 1.0 in all cases. Identification of other terpenes was based on data from a compound library of The National Institute of Standards and Technology (NIST. 05), built using authentic reference substances, with percentage of similarity above 90%.

Table 1. Outline of trials specifying chemical treatments, concentrations and expected response of adjuvants tested in young plants of *Araucaria angustifolia* (SuA: sulphuric acid; SA: salicylic acid; SNP: sodium nitroprusside; JA: jasmonic acid; ET: Ethrel®). All experiments were independently repeated twice, and each treatment consisted of five different plants with stems wounded at the insertion of five leaves.

treatments	concentration	purpose/expected response
trials of individual chemical stimulators		
SuA	0, 1, 5 and 10% (v/v)	Determine the optimal concentration of chemical stimulators for promoting resin exudation
SNP	0, 50 and 100 mM	
SA	0, 10, 50 and 100 mM	
ET	0, 50, 100 and 150 mM	
trials of combined chemicals stimulators		
SuA + SA	5% + 100 mM	Evaluate if combined chemical stimulators have a different effect on resin exudation
SuA + SNP	5% + 100 mM	
SuA + JA	5% + 100 mM	
SuA + ET	5% + 150 mM	
ET + JA	150 + 100 mM	
trials of chemical stimulators combined with ethylene action inhibitor (SN)		
ET + SN	150 + 20 mM	Investigate possible inhibitory effect of silver nitrate (SN), an ethylene action inhibitor, on resin exudation
JA + SN	100 + 20 mM	

Statistical analyses

To address if the different chemical treatments significantly influenced resin yield, data were submitted to ANOVA followed by Tukey tests. For data sets without variance homogeneity even after conventional data transformation protocols, non-parametric Welch's ANOVA was applied followed by Dunnett's C. For comparisons of two treatments in monoterpene concentrations, a Mann–Whitney test was applied due to lack of normality of the data set. In every case, $P \leq 0.05$ was used. Tests were done using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Exogenous application of SuA induced resin exudation from the wound site in the stems of *A. angustifolia* seedlings, up to 3.7-fold increase compared to controls (Table 2). Aliquots of 1%, 5% and 10% SuA were not significantly different in terms of resin exudation; however, 10% caused visible toxicity at the application site, *i.e.*, browning and subsequent drying of the stem. The apical region of the stem yielded more resin; middle and basal portions did not differ statistically (Fig. 1). Thus, a 5% SuA concentration and applications to the apical region were chosen for subsequent experiments.

Seedlings treated with 50 and 100 mol·m^{-3} SA showed higher resin exudation compared to the control, increasing yield 2.4- and 3.7-fold, respectively (Table 2). A similar response occurred in treatments with SNP, which at 50 and 100 mol·m^{-3} promoted yield increases of 3.3- and 4.9-fold, respectively (Table 2). The largest stimulations of resin production were obtained with JA (100 mol·m^{-3}) and Ethrel® (ET; 150 mol·m^{-3}), increasing 7.3- and 35.0-fold, respectively, when compared to the yield of control plants (Table 2).

In addition to the variation in the amount of resin produced between treatments, a large variation between treatments was observed for the length of time of resin exudation (Figure S1). Whereas the control treatment had exudation of resin for only 1 day after elicitation, the other treatments continued to exude resin after 1 week, even though that occurred only in a few individuals of some treatments (SuA, SA and SNP). After 2 weeks, ET and JA treatments continued exuding resin in large

Table 2. Resin production (means ± SE of means) by young plants of *Araucaria angustifolia* 3 weeks after being treated once with one of different concentrations of SuA (sulphuric acid – % v/v), SA (salicylic acid – mM), SNP (sodium nitroprusside – mM), JA (jasmonic acid – mM) and ET (Ethrel® – mM) at wound sites.

treatment	concentration	resin (mg)
sulphuric acid	0%	3.0 ± 0.0006 b
	1%	7.2 ± 0.001 ab
	5%	9.2 ± 0.001 a
	10%	11.4 ± 0.001 a
salicylic acid	0 mM	4.1 ± 0.5 c
	10 mM	6.8 ± 0.7 bc
	50 mM	10 ± 1.3 ab
	100 mM	14.8 ± 1.7 a
sodium nitroprusside	0 mM	3.1 ± 0.4 b
	50 mM	10.2 ± 1.3 a
	100 mM	15.1 ± 1.2 a
jasmonic acid	0 mM	3.4 ± 1.2 c
	50 mM	15.9 ± 1.4 b
	100 mM	24.9 ± 2.5 a
ethrel	0 mM	2.6 ± 0.2 c
	50 mM	12.9 ± 0.8 b
	100 mM	16.1 ± 1.2 b
	150 mM	72.9 ± 7.2 a

Different letters indicate significant difference by Tukey's test ($P \leq 0.05$; within SuA and SA experiments) or Dunnett's C test ($P \leq 0.05$; within SNP, JA and ET experiments).

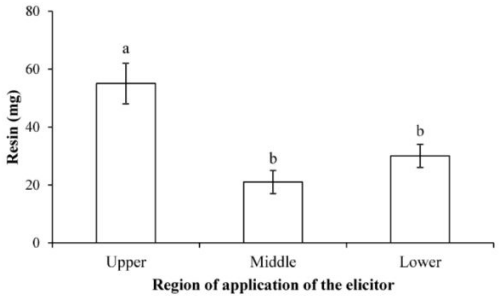

Fig. 1. Resin production in different portions of stems of *Araucaria angustifolia* treated with sulphuric acid (SuA, 5% v/v) at the wound site. Different letters above bars indicate significant difference by Dunnett's C test ($P \leq 0.05$). Lines on top of bars are SE of means.

quantities and in almost all plants analysed. By the third week, only a few plants treated with ET still showed resin exudation. In addition to observations of resin exudation in the stems, we also observed exudation at leaf tips.

The effect of the application of SuA plus elicitors (SA, JA, ET and SNP) was evaluated. The addition of SuA induced an increase in resin yield compared to the control, but there was no significant impact of SuA addition on resin yield induced by the different treatments (Fig. 2).

The addition of the ethylene action inhibitor, silver nitrate ($AgNO_3$), reduced the production of resin induced with both JA (29%) and ET (38%), but the decrease was not significant. When the two elicitors were applied together, there was an increase of 1.39-fold, compared to treatment with ET alone, and 2.29-fold compared to JA alone in resin yield, the latter being significant (Fig. 3).

The monoterpene composition of resin induced with the various treatments changed considerably in quantitative terms. The compounds analysed contained increased concentrations in all elicited resin treatments compared to those observed in control resin (Table 3). Camphene was the compound with higher accumulation after elicitation, showing an increase of 45-fold in the SuA treatment compared to the control. In ET, JA, ET + JA and SNP treatments the increase was 42-, 30-, 18- and 4-fold higher than the control, respectively. Limonene had the second highest accumulation, with increases of 14-, 11-, 11-, 10- and 2-fold in ET, SuA, JA, ET + JA and SNP treatments, respectively, compared to controls. α-Pinene concentration was increased by all treatments, and significant differences were observed with SuA, SNP and ET + JA. Upon SuA, JA and ET elicitation, β-pinene concentrations increased most compared to controls; these increases were statistically significant for SuA and JA. SuA, JA and ET increased the concentration of β-pinene so that it reached values close to those of α-pinene. Resin analysis also revealed a number of additional terpenes, particularly the sesquiterpenes germacrene, caryophyllene, bisabolol and elemene, as well as the monoterpene myrcene (Table S1).

DISCUSSION

Based on *Pinus* biology and resin-tapping practices published data, several chemical elicitors of resin exudation have been evaluated as potential stimulators of bark resin production in young plants of *A. angustifolia*. In spite of relevant differences, such as the presence of resin canals only in bark in *Araucaria versus* bark and wood canals in *Pinus*, as well as the large evolutionary distance between Pinaceae and Araucariaceae, essentially all of the chemical elicitors tested promoted resin exudation. In addition, to the best of our knowledge, stimulation of conifer resin production by NO was shown for the first time. Specificities of *A. angustifolia* reaction to the elicitors were mostly restricted to dose–response differences.

A concentration of 5% SuA was sufficient to cause increased resin production (Table 2), unlike observations for *Pinus elliottii* trees, where concentrations of 50% and 20% SuA were more efficient for the production of resin (McReynolds & Kossuth 1985; Rodrigues *et al.* 2008). This optimal concentration difference may be a consequence of age or species differences between plants used in these studies. In studies with *P. elliottii*, the plants were 23–60 years old, whereas in the present work young plants of 3–4 years old where used. The highest SuA (10%) concentration evaluated caused necrosis (browning) in the area of application, probably as a result of higher sensitivity of younger tissues. Signalling molecules, however, can induce resin production with less damage.

In fact, SA elicitation also promoted an increase in resin production (Table 2). This result is probably related to simulation of pathogen attack through SA application, as proposed for *Pinus* spp. (Rodrigues & Fett-Neto 2009). SA stimulates oxidative stress and the activation of defence-related genes, including those of secondary metabolism (Alvarez 2000; Shah 2003; Krajnc *et al.* 2011). In contrast, in *Pseudotsuga menziesii* and *Sequoiadendron giganteum*, methyl salicylate (10, 25, 50 or

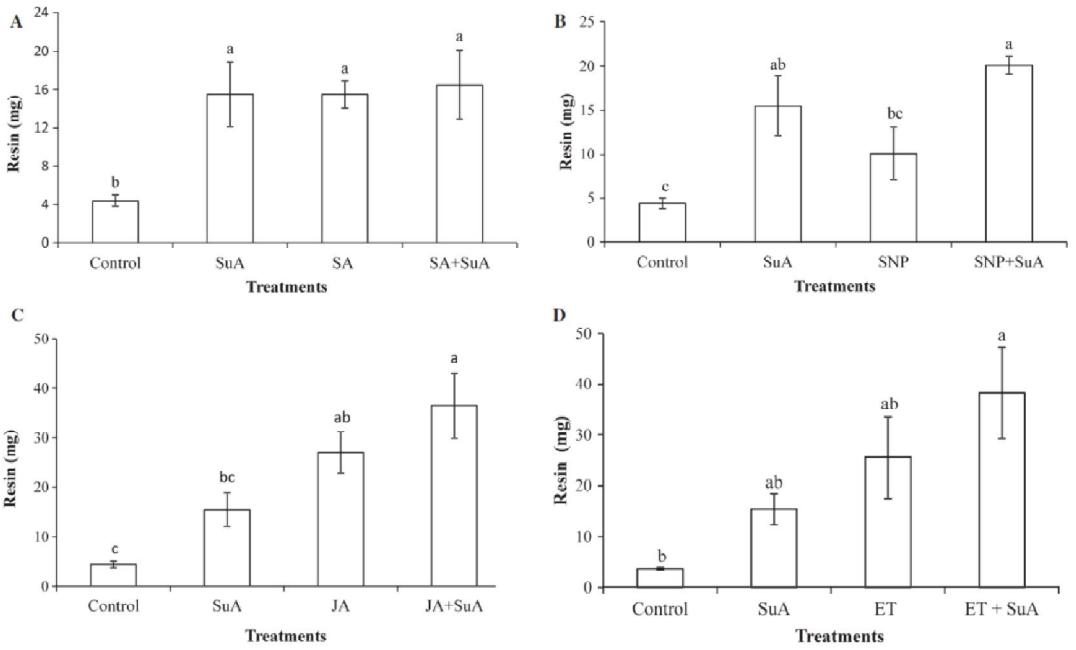

Fig. 2. Resin production in plants of *Araucaria angustifolia* treated with (A) salicylic acid (SA, 100 mol·m^{-3}), (B) sodium nitroprussiate (SNP, 100 mol·m^{-3}) (C) jasmonic acid (JA, 100 mol·m^{-3}) or (D) Ethrel® (ET, 150 mol·m^{-3}) at the wound site, alone or combined with sulphuric acid (SuA, 5% v/v). Different letters above bars indicate significant difference by Tukey's test ($P \leq 0.05$). Lines on top of bars are SE of means.

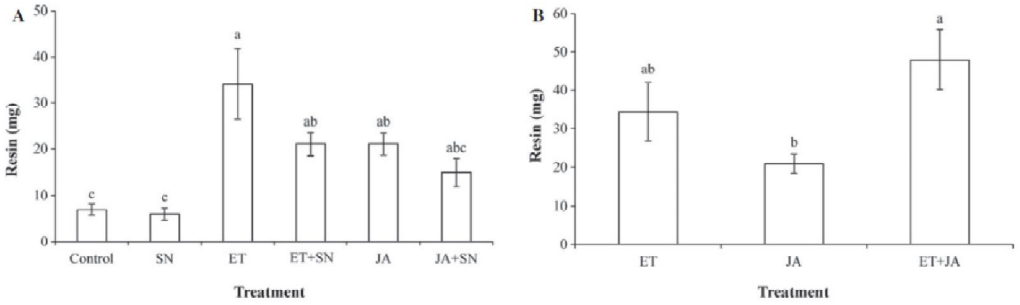

Fig. 3. A: Resin production in plants of *Araucaria angustifolia* treated with silver nitrate (SN, 20 mol·m^{-3}), Ethrel® (ET, 150 mol·m^{-3}), jasmonic acid (JA, 100 mol·m^{-3}) at the wound site, alone or combined with silver nitrate. B: Effect of Ethrel® (ET, 150 mol·m^{-3}), jasmonic acid (JA, 100 mol·m^{-3}) or combined application on resin yield. Different letters above bars indicate significant difference by Dunnett's C test ($P \leq 0.05$). Lines on top of bars are SE of means.

Table 3. Concentration (%) of monoterpenes in young *Araucaria angustifolia* resin, expressed on a weight basis (mean concentration ± SE).

	concentration (%) of monoterpenes			
treatment	α-pinene	camphene	β-pinene	limonene
control	0.48 ± 0.03	0.01 ± 0.004	0.14 ± 0.005	0.06 ± 0.001
SNP	0.83* ± 0.04	0.04* ± 0.005	0.09 ± 0.03	0.14* ± 0.01
SuA	1.55* ± 0.2	0.45* ± 0.2	1.19* ± 0.4	0.66* ± 0.3
ET	0.83 ± 0.1	0.42* ± 0.1	0.52 ± 0.1	0.85* ± 0.2
JA	0.9 ± 0.2	0.3* ± 0.08	1.02* ± 0.2	0.65* ± 0.1
ET + JA	1.07* ± 0.2	0.18* ± 0.08	0.32 ± 0.07	0.59* ± 0.01

Significant differences compared to control are marked with an asterisk (Mann–Whitney, $P \leq 0.05$).

100 mol·m^{-3}) had no apparent effect on resin accumulation (Hudgins & Franceschi 2004), suggesting species-dependent responses or reflecting variations in experimental settings. Changes in presence and concentration of other molecules that can affect SA metabolism and action could contribute to the discrepancies observed. On the other hand, these cross-regulators may also contribute to expand the array of potential resin elicitors.

Nitric oxide has been reported to regulate SA biosynthesis and signalling (Feechan *et al.* 2005; Zottini *et al.* 2007; Mur *et al.* 2012). Studies have demonstrated the involvement of NO in many biological processes in plants. It acts as a signal molecule in plant defence mechanisms or as a compound with hormonal properties, affecting growth and development functions (Yu *et al.* 2014). Our results showed that SNP, an NO donor, increased the yield of resin in *A. angustifolia* more than five times compared to the control (Table 2), raising the possibility that NO may take part in resinosis signalling. NO has been shown to promote the expression of JA and ethylene biosynthetic enzymes (Mur *et al.* 2013).

Jasmonic acid and its methyl ester, MeJA, are capable of inducing chemical and anatomical defence responses in conifers (Franceschi *et al.* 2002; Martin *et al.* 2002; Hudgins *et al.* 2003, 2004; Zulak & Bohlmann 2010; Moreira *et al.* 2012). In the present study, we observed an increase of 7.3-fold in resin yield with 100 mol·m^{-3} JA compared to control (Table 2). This result is consistent with reports of induced resin accumulation in stems of *Araucaria araucana, A. heterophylla, Pinus pinaster* and *Picea abies* after application of 100 mol·m^{-3} MeJA (Franceschi *et al.* 2002; Hudgins *et al.* 2004).

Like JA, ethylene also plays a major role in conifer defence responses. Ethylene regulates differentiation of traumatic resin ducts and promotes resin synthesis (Yamamoto & Kozlowski 1987; Rodrigues *et al.* 2011). Exogenous application of Ethrel® has also been shown to induce enhanced resin exudation in Cupressaceae (Kusumoto & Suzuki 2001). ET application in *A. angustifolia* caused an increase of about 30-fold in resin production compared to untreated plants, and the flow of exudate continued until the third week after application. These results are in agreement with the expected long-term action of ET (Table 2). Although the differences in exudation time may be influenced by genetic characteristics of the individual plants, the effect of the treatments was predominantly based on the occurrence of longer exudation periods only after the application of specific chemical adjuvants. Resin production yields were consistently induced by adjuvants with or without co-application of SuA.

The addition of SuA to SA, JA, ET or SNP did not result in a significant increase in resin yield during the time points sampled (Fig. 2). In adult trees of *Pinus elliottii* and *P. palustris* combinations of SuA and ET had a higher stimulant effect compared to ET alone (McReynolds & Kossuth 1985). These results may be influenced by age, species and concentration range tested for the adjuvants. Although SuA co-application did not result in significant changes in resin exudation, the combination of other adjuvants, particularly those that have a morphogenetic role (*e.g.* JA and ET), may have a magnified impact on resinosis.

The interaction between JA and ethylene is complex. MeJa has been shown to induce ethylene production in several species (Fan *et al.* 1998; Farmer *et al.* 2003; Hudgins & Franceschi 2004). In *Arabidopsis*, responses to different pathogens showed a synergistic effect of JA and ethylene for induction of defence-related genes (Xu *et al.* 1994). In the present study, a clear synergism was not apparent. Although resin production induced by JA alone was increased if combined with ET application, resin yield of ET and ET + JA were equivalent (Fig. 3B). Such a response is in line with the proposed sequential action of JA leading to ethylene production in the signal transduction of resinosis (Franceschi *et al.* 2005; Rodrigues-Corrêa & Fett-Neto 2012).

The ethylene action inhibitor silver nitrate (SN) caused an apparent decrease of resin production induced by application of JA or ET, although not to a significant extent, possibly due to the use of a relatively low concentration (Fig. 3A). Studies with *Pseudotsuga menziesii* showed that the JA-induced defence response is mediated by ethylene (Hudgins & Franceschi 2004). Marginally lower resin yield in *A. angustifolia* treated with JA or ET upon co-application of SN may further indicate that a similar interaction among these regulatory molecules occurs in the control of *A. angustifolia* resinosis.

In addition to resin yield, another important aspect of resinosis elicitors is their impact on terpene composition. Monoterpene concentration was increased by all treatments examined (SNP, SuA, ET, JA, ET + JA). Camphene and limonene had higher accumulation after elicitation. In the control treatment, the concentration of α-pinene was higher than β-pinene (Table 3). Upon SuA, ET and JA elicitation, β-pinene concentrations increased the most compared to controls, bringing the concentration of this isomer close to that of α-pinene. The same result was observed in Norway spruce, in which increases in many monoterpenoids in wood and bark occurred after MeJA treatment. In wood, the main modification was the proportion of β-pinene to α-pinene (the two monoterpene resin constituents in the wood found in about equal amounts). Limonene accumulation was also substantially induced following MeJA treatment (Martin *et al.* 2002). Concentrations of α-pinene and β-pinene were elevated in stems of *Pinus radiata* seedlings after MeJA application (Gould *et al.* 2009). Accumulation of all three classes of resin terpenoids, mono-, sesqui- and diterpenes, was strongly increased in inner stem tissues of *Picea abies* upon MeJA treatment (Martin *et al.* 2002).

Pinaceae and Araucariaceae have been separate evolutionary lines for a long time and possess significant differences in evolutionary dynamics (Leslie *et al.* 2012). However, young plants of *A. angustifolia* displayed regulatory features of resin exudation that are similar to those of *Pinus* species. Considering the evolutionary distance between these genera, this is somewhat surprising, suggesting that regulation of resin production evolved relatively early in gymnosperm history, perhaps before the geographic separation and speciation of the North and South American conifers. Alternatively, control of resin exudation may be the result of independent events that converged to a comparable regulation profile.

A study on the anatomical and histochemical changes caused by MeJA application to unwounded stems of young trees of various conifer species showed that this metabolite acts as a ubiquitous signalling molecule for up-regulating terpenoid- and phenylpropanoid-based defences. However, *Araucaria araucana* and *A. heterophylla* were devoid of traumatic xylem resin duct responses, but activated and induced new axial

phloem resin ducts, whereas the Pinaceae examined (which did not include *Pinus* spp.) had constitutive and inducible resin xylem ducts and only constitutive phloem resin structures (Hudgins *et al.* 2004). Similar to *A. araucana* and *A. heterophylla*, *A. angustifolia* lacks wood resin canals and resinous axial tracheids (Esteban *et al.* 2004), displaying resinous canals in the bark (FAO 1986). Despite these anatomical differences in the main tissues involved in resin production, our study further supports the idea that regulatory signals in resinosis of different conifers are conserved, even in combination with the wound response.

Araucaria angustifolia resin may represent a relevant source of terpenes of industrial interest. Camphene is used in the production of pharmaceuticals, plasticisers, repellents, explosives and fragrances. Limonene is employed in solvents, cleaning agents, flavourings, fragrances, insect attractants and perfumes. Pinenes are applied in the production of repellents, insecticides, solvents, perfumes, fragrances, flavourings, plasticisers, antiviral and antimicrobial agents. The sesquiterpenes germacrene, caryophyllene, bisabolol and elemene, as well as the monoterpene, myrcene, have a number of applications in the food, chemical, pharmaceutical and biofuel industries (Rodrigues-Corrêa *et al.* 2012, 2013; Zou *et al.* 2013).

In conclusion, control of resinosis in *A. angustifolia* shares many features with that of the more derived genus *Pinus*. Resin extraction from *A. angustifolia* may represent a viable alternative for faster economic returns from plantations of this slow-growing species, contributing to its sustainable use in reforestation programmes. Further studies are needed for characterisation of the most affordable chemical elicitors identified in this work for the production of *A. angustifolia* resin in adult trees. Future investigations addressing the molecular basis of resinosis in this unique species may shed additional light on the regulation of this complex defence response.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time course of resin exudation by stems of *Araucaria angustifolia* plants submitted to different treatments of single local stimulation at the time of wounding (SA – salicylic acid 100 mol·m^{-3}, SNP – sodium nitroprussate 100 mol·m^{-3}, SuA – sulphuric acid 5% (v/v), JA – jasmonic acid 100 mol·m^{-3}, ET – Ethrel® 150 mol·m^{-3}).

Table S1. Additional terpenes of economic interest identified by comparison with a library of compounds with the corresponding areas of chromatograms.

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SUPPORTING INFORMATION

(Plant Biology doi:10.1111/plb.12298)

Table S1. Additional terpenes of economic interest identified by comparison with a library of compounds with the corresponding areas of chromatograms

Terpenes	Control	SNP	SuA	JA	ET	ET+JA
Triciclene	1.03	2.44	2.16	3.09	3.22	3.76
Myrcene	2.7	2.53	2.71	1.62	2.91	2.19
γ-Elemene	14.5	13.43	11.78	12.02	7.85	10.5
β-Elemene	0.74	-	1.85	1.73	0.86	1.19
Copaene	2.3	2.13	1.59	1.79	1.51	1.27
Caryophyllene	2.96	3.08	2.71	1.73	1.85	2.2
Humulene	2.4	1.52	1.5	1.54	1.48	1.45
γ-Muurolene	1.1	-	5.53	1.36	0.87	0.82
Germancrene - D	47.6	39.99	40.79	35.19	27.68	29.61
Δ-Cadinene	1.1	1.08	1.27	1.18	1.25	0.82
Spatulenol	0.91	-	-	1.12	1.47	1.06
α-Bisabolol	8.5	7.43	9.53	1.18	9.02	7.55

Identification of terpenes was done through the *NIST 05* Library with a percentage of similarity above 90%. Values refer to the relative percentage of the area.

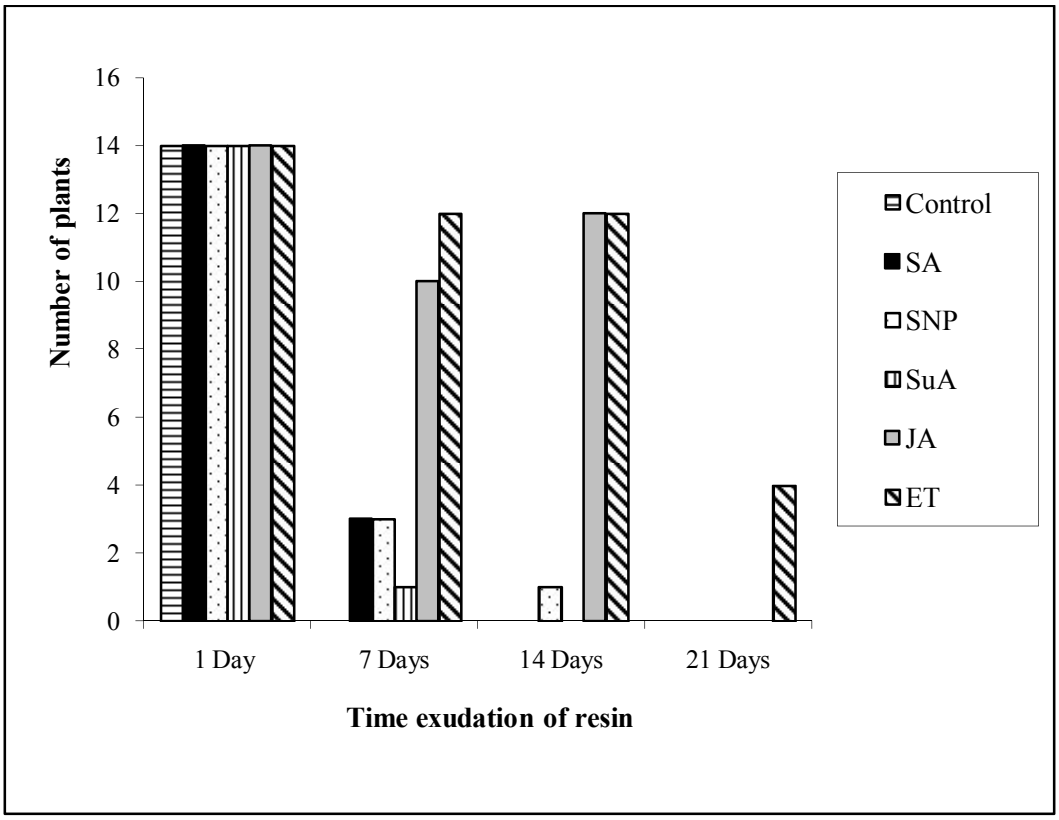

Fig. S1 – Time course of resin exudation by stems of *Araucaria angustifolia* plants submitted to different treatments of single local stimulation at the time of wounding (SA – salicylic acid 100 mM, SNP – sodium nitroprussiate 100 mM, SuA – sulfuric acid 5% (v/v), JA – jasmonic acid 100 mM, ET – ethrel150 mM).

CAPÍTULO II

Artigo a ser submetido ao periódico

Plant Biology

GROWTH AND METABOLIC RESPONSES OF A COMMERCIAL EUCALYPT HYBRID EXPOSED TO COMBINATIONS OF TEMPERATURE AND CO_2 UNDER NITRATE SUPPLY

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Running head: **Eucalypt hybrid response to high temperature and CO_2**

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Key Words: *Eucalyptus*, temperature, CO_2, climate change, nitrate, photosynthesis, metabolism

ABSTRACT

The physiological responses of a commercial hybrid of eucalypt (*Eucalyptus grandis* W. Hill X *E. urophylla* S.T. Blake) having nitrate as sole N source to high CO_2 and heat, and a combination of both, were investigated. We tested the hypothesis that under moderate temperature there would be a limitation in the use of excess CO_2 by C3 plants due to difficulties in assimilating nitrogen as a result of low reducing power, a situation that could be partly compensated by photorespiration under high temperature and no water restriction, because of a recovery of organic acid availability, reducing power, and consequent nitrate assimilation. For the study of the effect of CO_2 elevation and high temperature in eucalypt, the treatments evaluated in a growth chamber were control conditions (25/20ºC (day/night), 379 ppm CO_2) elevated CO_2 (EC, 730 ppm CO_2, 25/20ºC), elevated temperature (ET, 30/25ºC, 379 ppm CO_2), and combination (EC+ ET, 30/25ºC, 730 ppm CO_2). Several indicators were monitored, including: growth and photosynthetic parameters, chlorophyll fluorescence, as well as the content of carbohydrates, phenolics, anthocyanins, soluble proteins, and nitrate reductase activity. The main results were the better photosynthetic performance and growth of plants under ET and EC+ET, what was not observed for EC only, partially supporting the hypothesis that reducing power limits the benefit and effective use of CO_2 by C_3 plants under conditions not leading to photorespiration. Thereby, this indicates the possibility of a compensatory response of high temperature to overcome the difficulties of nitrate assimilation under water availability in eucalypt hybrids, particularly under high CO_2 in future climatic scenarios.

INTRODUCTION

The increasing deforestation, forest burning, use of fossil fuels and energy demand have intensified the emission of CO_2 into the atmosphere and cause climate change worldwide. The concentration of atmospheric CO_2 increased from 280 to 390 μmol mol^{-1} since 1800, and is predicted to reach between 530-970 μmol $mol^{-1}$by the end of the century, resulting in an increase in temperature ranging from 0.3 - 1.7º C to 2.6 - 4.8° C on a global scale (IPCC, 2014). Drought spells and floods are predicted for different parts of the globe. In Brazil, for example, the south may experience higher rainfall and floods, whereas in the north and northeast water shortage may be a major limitation (IPCC, 2014). It is believed that plants could reduce the concentration of atmospheric CO_2 by photosynthesis for their conversion in carbohydrates and other organic components, promoting an increase in the growth rate of plants. However, the exact potential for carbon mitigation remains uncertain.

Biomass accumulation in plants depends on several factors, including leaf area, photosynthetic capacity of individual leaves and the availability of nutrients in the soil. Biomass gain and plant growth are strongly related to photosynthetic activity. Plant growth, measured by biomass accumulation, is a result of the difference between carbon gains via photosynthesis, and carbon losses by respiration, photorespiration, release of volatile carbon compounds and production of exudates. The maximum plant efficiency for converting solar energy into biomass is relatively low, generally less than 2% because various losses occur during the process of transformation of radiant energy in biomass (Eamus, 1996). C3 plants exposed to high CO_2 concentrations have ample supply of this gas in carboxylation sites of ribulose 1,5-bisphosphate carboxylase / oxygenase (Rubisco); in this situation, photosynthetic rate is limited by biochemical reactions connected with the transport of electrons in the photochemical phase of photosynthesis.

Temperature affects photosynthetic assimilation of CO_2 in a number of ways. Temperature can influence the carbon partitioning between organs in growth. Temperatures between 20-35ºC favor the metabolism of sink organs, which increases the demand for carbohydrates with increasing temperature. Thus, there is a reduction in the accumulation of carbohydrates in leaves, which can avoid acclimation to CO_2. However, temperature extremes have negative effects on translocation of carbohydrates of leaves to the sink because the

phloematic flow is reduced in these conditions, and the accumulation of carbohydrates in the leaves can contribute to CO_2 acclimation (Wolfe *et al*., 1998; Caliman, 2008).

Temperature also influences photorespiration, which increases under heat, mainly reducing the diffusion of CO_2 and increasing the O_2/ CO_2 ratio in the mesophyll. As result of competition between O_2 and CO_2 by Rubisco enzyme, net photosynthesis of C3 plants is engaged when the temperature reaches values above 30 ° C (Salisbury & Ross, 1992).

Photosynthetic capacity strongly depends on nitrogen nutrition, because the photosynthetic system demands a large amount of N to assemble Rubisco and other proteins involved in the process. The addition of nitrogen to the soil may increase the effect of CO_2 on plant productivity, causing a strong interaction of CO_2 x N in the total biomass of the shoot (Makino, 2000; Langley & Megonigal, 2010).The increased CO_2 assimilation inhibits NO_3^-, and this decrease in the nitrogen content in the plants plays a role in CO_2 acclimation phenomenon of C_3 plants. Thereby, the importance of reducing and assimilating of nitrate for plant life is so important that it has been considered similar to the reduction and assimilation of carbon dioxide in photosynthesis (Marschner, 1986),

Due to contradictory existence of data in the recent literature, this work aims to evaluate the individual and combined effects, of increased temperature and carbon dioxide concentration on the growth and physiological responses of one of the most popular clones used in forestation in Brazil, the hybrid *Eucalyptus grandis* x *E. urophylla* (Myrtaceae). We tested the hypothesis that in C_3 plants, especially trees, the increased photorespiration, induced by high temperature, may compensate the limitation ofnitratereduction for assimilation, which is caused by poor reducing power and low organic acids prevailing under high CO_2 environments.

MATERIALS AND METHODS

Plant material and treatments

Hybrid *Eucalyptus grandis* x *E. urophylla* (urograndis) plants were kindly supplied by CMPC Celulose Riograndense (Barra do Ribeiro, Rio Grande do Sul, Brazil) with approximately

three months of age. For each experiment 120 seedlings were transplanted in plastic pots (700 mL) containing expanded vermiculite and commercial substrate peat fertile (Garden Plus®) (1:1). The substrate was fertilized with nutrient solution containing nitrate as only N source(Table S1) until the container capacity was reached. On the same day, trees were transplanted and after one week of adaptation, they were transferred to a growth chamber (PGR15 Conviron, Canada) where they remained for four weeks under different temperature and [CO_2] treatments, control conditions (25/20ºC (day/night), 379 ppm CO_2) elevated CO_2 (EC, 730 ppm CO_2, 25/20ºC), elevated temperature (ET, 30/25ºC, 379 ppm CO_2), and combination (EC+ ET, 30/25ºC, 730 ppm CO_2). Maintained under 60% relative humidity, and approximately 500 µmol $m^{-2}s^{-1}$ photosynthetic active radiation (PAR), photoperiod of 12 h/light. Plants were irrigated daily and rotated within growth chamber every three days to minimize potential effects of position on plant performance.

Measurement of plant growth

Three harvests were done for each treatment: in the deployment day (day 0), 7 and 14 days after the experiment started. In each time point, we measured the length of main stem, the number of leaves per plant and the relative growth rate (RGR), both on fresh and dry mass basis of shoots and roots (calculated as final mass minus the initial, divided by the initial mass), for 10 plants of each treatment. The dry biomass of plant parts (shoot and roots) were determined after drying in an oven at 60°C until constant weight.

Leaf gas exchange measurements

Net photosynthetic rate (A µmol mol^{-1}), stomatal conductance (g_s, mol $m^{-2}s^{-1}$), transpiration rate (E, mol $m^{-2}s^{-1}$), instantaneous transpiratory efficiency (A/E, mmol mol^{-1}) and water use efficiency (WUE: A/gs) were measured at the beginning (day zero) and end (14^{th} day) of the experiment. At the end of the experimental period (21^{st} day), additional parameters were obtained: rubisco carboxylation rate (V_c µmol $m^{-2}s^{-1}$), maximum rate of electron transport (J_{ETR} µmol $m^{-2}s^{-1}$), triose-phosphate utilization (TPU µmol $m^{-2}s^{-1}$) and leaf dark respiration (Rd µmol $m^{-2}s^{-1}$), assessed from CO_2 assimilation rates in relation to leaf internal CO_2 concentration (A/C_i)

curves, using a portable photosynthesis system model Li-6400 (Li-Cor, Lincoln, NE, USA). The measurements were taken in the youngest fully expanded leaf, on five seedlings per treatment. At the time of the measurements chamber conditions were the same as those used in the respective treatments.

Chlorophyll fluorescence

Chlorophyll *a* fluorescence was measured at the respective temperatures of the treatments, in the experiment deployment day (day 0), 7 and 14 days after beginning of the experiment. Measurements were obtained for fully expanded leaves. Ten leaves per treatment were measured using a Chlorophyll Fluorometer Opti-Sciences OS-30P (Hudson, NH, USA). From these measurements, another set of parameters were determined: maximum photochemical efficiency of photosystem II (Fv/Fm), ratio of variable to minimal chlorophyll fluorescence (Fv/Fo), quantum yield of dissipation (Fo/Fm), performance index (Pi_{ABS}) and total performance index (Pi_{total}).

Metabolite analysis

For each treatment, four samples, each consisting of five expanded leaves of different plants were harvested. After harvest, samples were immediately wrapped in aluminum foil, immersed in liquid nitrogen and then were stored in ultrafreezer at -80°C until analysis.

Starch and total soluble hexoses

Leaf tissue samples (30mg) were ground in liquid nitrogen and hexoses and starch were extracted according to the methods of Dubois *et al*. (1956) and McCready et al (1950), respectively. For the extraction of soluble sugars, samples were incubated in 80% ethanol in a boiling water bath for 15 min and then were centrifuged at 18000 xg, at 4°C for 10 min. The supernatant was recovered and the sediment was re-extracted.

For starch extraction, the pellet was used. Double distilled water (250µL) and 36.5% perchloric acid (320 µL) were added to the pellet. Starch was extracted for 15 min in a sonic

bath. The samples were then centrifuged at 18000 xg, at 4°C for 10 min. The supernatant was recovered and the sediment was re-extracted. From the combined extracts, 200 µL (diluted 20x) were used for reaction. For quantification of total starch, the samples were recorded in a microplate spectrophotometer SpectraMax - M2 (Molecular Devices) at 630 nm.

For quantification of total soluble hexoses 75µL of extract were incubated for 20 min at room temperature, with 80% ethanol (125µL), 5% phenol (200 µL) and concentrated sulfuric acid (1mL). The samples were recorded in a microplate spectrophotometer SpectraMax - M2 at 490 nm. Standard curves for quantifying starch and hexoses were prepared with D-glucose.

Total protein

Quantification of total protein was by the Bradford method (1976). Leaf tissue samples (330mg) were ground in liquid nitrogen and extracted in 2 mL of 0.2 M phosphate buffer pH 7.0. The samples were centrifuged at 18000 xg, at 2°C for 30 min. The supernatant was recovered and the sediment was dried in an oven to determine dry weight. For quantification of total soluble protein, extracts were incubated for 15 min with the Bradford reagent and recorded in a microplate spectrophotometer SpectraMax - M2 at 595nm. A standard curve was prepared with BSA (bovine serum albumin).

Total soluble phenolics

Soluble phenolics were extracted as described by Fett-Neto *et al.* (1992). Leaf tissue samples (30 mg) were ground in liquid nitrogen and total phenolics were extracted for 30 min in a sonic bath in 700 µL ice cold 0.1 M HCl. The samples were centrifuged at 18000 xg, at 4°C for 10 min. The supernatant was recovered and the sediment was re-extracted. The combined extracts were made up to 1.5 mL with 0.1 M HCl. For the reaction, 200 µL of 20% (w/v) $NaCO_3$ and 100 µL of Folin-Cicalteau reagent (Sigma, USA) were added to the extracts. The samples were mixed and incubated in a boiling water bath for 1 min and then immediately chilled in ice. After cooling, the solutions were centrifuged at 18000 xg, at 4°C for 5 min, The supernatant was diluted in a proportion of 1:1, and recorded in a microplate spectrophotometer SpectraMax - M2 at 750 nm. A standard curve was prepared with pyrogallol.

Anthocyanin

Total anthocyanin content was estimated by the method of Chatterjee *et al.* (2006). Samples (30 mg) were ground and extracted in 1 ml of acid methanol (MeOH-concentrated HCl 99:1, v:v) overnight, in the dark, under constant shaking. The samples were centrifuged at 18000 xg, at 4°C for 5 min and volume was made up again to 1 mL. Chloroform (600 µL) was added to the samples (600 µL) and the blend was mixed for 10s. Double distilled water (300 µL) was then added, and samples were mixed for 10s. After phase separation, absorbance of the aqueous (upper) phase was recorded at 530 nm in a microplate spectrophotometer SpectraMax - M2. The tissue pellets were dried at 60°C until constant weight. Anthocyanin content was expressed as absorbance per extracted dry weight

Chlorophyll

Total chlorophyll was determined by the method of Ross (1974). Samples (30 mg) were ground in liquid nitrogen in the dark and extracted in acetone 85% by sonication. After centrifugation a20000 xg for 15 min, the absorbance of the extract at 645 and 663 nm was recorded in a microplate spectrophotometer SpectraMax - M2. The pellet was dried for a week at 60°C and the extracted dry mass determined. Chlorophyll content was expressed on an extracted dry weight basis.

Nitrate reductase (NR) activity

Total NR activity was measured following the method of Queiroz *et al.* (1991) with modifications. Leaf tissue samples (200 mg) were ground in liquid nitrogen and mixed with 1.5 mL of incubation solution containing 0.1N phosphate buffer pH 7.5, 0.02M KNO_3, 2% propanol and 5% Triton X 100. The samples were extracted for 5 min in a sonic bath, and then centrifuged at 18000 xg, at 4°C for 10 min. The supernatant was recovered and incubated in a water bath, at 30°C for 30 min in the dark and then immediately chilled in ice. Next, a 150 µL of a reaction solution (37.5µL of 1% sulphanilamide diluted in 3N HCl, 37.5µL of 0.02% *N*-(1-Naphthyl)

ethylenediaminedihydrochloride, 75µL of double distilled water) was added to aliquots of the samples (350µL), mixed, and incubated for 20 min. The mixtures were added in a microplate spectrophotometer SpectraMax - M2 at 540 nm. A standard curve was prepared from dilutions of a 37.7 µmol $NaNO_2$ stock solution. Enzyme activity was expressed as moles of NO_2^- .g^{-1} edw.h^{-1}.

Statistical analyses

The treatments were run separately because of chamber size and operational limitations. The experimental design was completely randomized, using ten replicates for growth analysis, thirty replicates for leaf gas exchange measurements, ten replicates for chlorophyll fluorescence analysis and four replicates for biochemical analysis. The data used for statistical analysis were all relative to the beginning of the respective trial - day 0 - to normalize treatments, given that the treatment tests were performed separately with different groups of plants.

Data were analyzed using analysis of variance (ANOVA) followed by Tukey test. For data sets without variance homogeneity, non-parametric Welch's ANOVA was applied, followed by Dunnett's C test. In every case, $P \leq 0.05$ was used. Tests were done using SPSS software 17.0 (SPSS Inc. Chicago, IL, USA).

RESULTS AND DISCUSSION

Growth analyses

The ET and EC+ET treatments showed higher number of leaves per plant, with increases of 7.3 and 4.4 times, respectively, compared to the control (Table 1). The highest average plant height was also observed in ET and EC+ET treatments, with increases of 1.26 and 1.11 times, respectively, compared to the control (Table 1). The EC treatment was not able to influence any of the growth parameters evaluated (Table 1).

The observed positive impact of higher temperatures in stem height and leaf number in the absence of water restriction is in good agreement with data obtained for other species. The final height of *Picea abies* seedlings increased in response to higher temperature (Sallas *et al*. 2003). In 150 day-old *E. Saligna* and *E. sideroxylon*, a greater leaf number was also observed under high temperature and elevated CO_2. At ambient and increased [CO_2], stem height was higher in high temperature treatment than in the ambient temperature for the same species (Ghannoum *et al*. 2010). Also in accord with the present work, growth parameters (height and diameter) in *E. urophylla* and in the hybrid *E. deglupta* x *E. camadulensis* were not influenced by elevated [CO_2], in spite of increasing net photosynthetic rate (Novriyanti *et al*. 2012).

In *E. radiata* under well watered conditions, dry mass of seedlings increased substantially in high [CO_2] and high temperature treatments compared to ambient CO_2 and temperature, on average 1.7-fold increase in dry mass, with a significant interaction between [CO_2] and temperature (Duan *et al*. 2014). Ghannoum *et al*. (2010) also recorded an increase in whole plant dry mass in the order of 44% higher in *E. saligna* and 74% higher in *E. sideroxylon* at elevated [CO_2] (640 μL L^{-1}) compared to ambient [CO_2]. At high temperature (30/22°C) and ambient CO_2 (400 μL L^{-1}), whole plant dry mass increased by 52% in *E saligna* and 37% in *E. sideroxylon* relative to the ambient temperature treatment. It is important to note that, in all of the above mentioned studies, eucalypt plants in basal treatments were treated with commercial fertilizers, with no nitrogen restriction or specific supply as nitrate only (Ghannoum *et al*. 2010; Novriyanti *et al*. 2012; Duan *et al*. 2014).

In the present work, in which nitrogen supply was provided as nitrate, elevated CO_2 did not differ from the control treatment, whereas leaf number was significantly higher in ET and ET+EC treatments, the same applying for FM of shoots (Table 1). Higher values in dry mass of shoots in ET and EC+ET treatments were also observed, as well as higher dry mass of roots in EC, although these increases did not reach statistical significance at 5% (Table 1).

CO_2 enrichment inhibits shoot nitrate assimilation in a wide variety of C_3 plants and this phenomenon can influence whole-plant growth (Bloom *et al*. 2012). Among the physiological mechanisms which may be responsible for the relationship between elevated atmospheric CO_2 concentrations and shoot NO_3^- assimilation is the first biochemical step of this ion assimilation, the conversion of NO_3^- to NO_2^- in the cytoplasm of leaf mesophyll cells. Photorespiration is the biochemical pathway in which the chloroplast enzyme Rubisco catalyzes the oxidation of the

high energy substrate RuBP rather than catalyzing its carboxylation through the C_3 carbon fixation pathway (Foyer *et al*. 2009). Photorespiration also stimulates the export of malic acid from chloroplasts and increases the availability of reduced nicotinamide adenine dinucleotide (NADH) in the cytoplasm, which fuels this first step of NO_3^- assimilation. CO_2 enrichment decreases photorespiration and thereby decreases the amount of reductant available to power NO_3^- reduction (Igamberdiev *et al*. 2001; Bloom *et al*. 2012).

Hence, it is possible that under high CO_2 and a predominant nitrate supply in the substrate, an increase in photorespiration promoted by higher temperatures may provide the necessary organic acids and reducing power to carry out nitrate assimilation. The higher growth data observed in the EC+ET treatment seems to support that assumption. In fact, plants used in a preliminary assay under EC, in which urea (at equimolar amount of N) was added after the chamber adaptation period (under regular CO_2 and nitrate) in comparison with a new application of nitrate itself, had higher growth rates on a dry mass basis for both shoots and roots (data not shown). Since eucalypts, like most plants, have readily available ubiquitous urease (Sirko & Brodzik 2000; Schwambach *et al.* 2014), the reduced nitrogen provided as urea allowed prompt N assimilation, relieving the need for reducing power that was required for nitrate assimilation. The fact the ET treatment also had higher growth responses may be the result of an improved use of ambient CO_2 by the same principle of 'secondary' benefits of photorespiration in nitrate assimilation.

Leaf gas exchange

Net photosynthetic rate (*A*) in EC+ET showed an increase of 1.79 times compared to control (for data on the relationship 7^{th} on day 0). Over time (data on the relationship 14^{th} on day 0), *A* decreased in all treatments, with ET and EC+ET showing higher values compared to that of the control. In both days evaluated, EC did not differ from control treatment (Table 2).

Elevated [CO_2] usually increases *A* and thereby the growth rate, as reported for *E. urophylla* and in the hybrid *E. deglupta* x *E. camadulensis* (at 760 µmol mol^{-1}) (Novriyanti *et al*. 2012). In *E. saligna*, *A* was increased by both elevated carbon and higher temperature, whereas in *E. sideroxylon A* responded only to elevated CO_2 (Ghannoum *et al*. 2010). In the present

study, there was no positive effect of elevated [CO_2] in *A* and, consistently, no effect on growth. On day 7, the combination EC+ET caused significant increase in *A*, reaching the highest values. This result may also be explained by the photorespiration benefits to nitrate assimilation discussed above, since photosynthetic rate is closely dependent on N assimilation.

Stomatal conductance (g_s) was higher in ET and EC+ET treatments, an increase of 2.15 and 2.50 compared to control at 7d. However, the stomatal conductance of EC did not differ from that of control. At 14d, the control treatment had the highest g_s average (Table 2). In *E. radiata* g_s was not affected by [CO_2] and temperature (Duan *et al.* 2014). In *E. saligna*, g_s decreased in the condition equivalent to EC+ET (640 µL L^{-1} CO_2, 30°C) treatment relative to control, whereas *E. sideroxylon* showed a slight increase (Ghannoum *et al*. 2010). In a hybrid of *E. deglupta* x *E. camadulensis*, g_s decreased under high CO_2 (Novriyanti *et al.* 2012).

Temperature can affect photosynthesis through leaf temperature, by defining the magnitude of the leaf to air vapor pressure difference, a key factor influencing stomatal conductance (Lloyd & Farquhar, 2008). As evaporative demand vapor pressure difference increases, stomata tend to close to reduce the rate of water loss through transpiration. Associated with this stomatal closure is a reduction in CO_2 assimilation rate *A* due to a reduction in the rate of supply of CO_2 to the chloroplast (Farquhar & Sharkey, 1982).

The analysis of the net photosynthetic rate time course showed highest rates in EC+ET and ET at 7d and of EC+ET at 14d. Throughout the observation period, the lowest values of A were recorded for EC and control. Stomatal conductance showed a similar profile, with an increase of g_s in control treatment at 14d (Figure S1).

ET and EC+ET had higher average transpiration rate (*E*), with an increase of 2.1 and 2.3 times compared to the control. Later, at 14d, the lowest *E* was observed in the EC treatment, ET and EC+ET being equivalent to the control (Table 2). According to Leakey *et al.* (2009) elevated CO_2 decreases *E* between 5 and 20% as stomata close in response to higher intercellular CO_2 concentration, a result that can also be seen in our work (reduction of 26% by 14d).

Regarding water use efficiency (WUE), EC did not differ statistically from the control and EC+ET. By 14d, the control average declined, being statistically lower than those of other treatments (Table 2). The instantaneous transpiratory efficiency (*A/E*) was higher in control at 7d. At 14d, *A/E* declined in the control, ET and had small decrease in EC+ET, remaining unchanged in the EC treatment (Table 2). In *E. radiata* WUE was higher in seedlings grown in

elevated [CO_2] (640 µL L^{-1}, 26°C) and in a treatment comparable to EC+ET (640 µL L^{-1}, 30°C) (Duan *et al.* 2014). Eamus (1991) also reported that plant WUE was substantially increased by high CO_2 even at high temperature.

Rubisco carboxylation rate (Vc) and maximum rate of electron transport (J_{ETR}) were higher in ET and EC+ET treatments, particularly in the latter (Figure 1 A and B). EC+ET also showed the highest rate of triose-phosphate utilization (TPU) (Figure 1C). Leaf dark respiration (Rd) was not significantly different among treatments (Figure 1D). Specific respiration rates are generally not reduced when plants are grown at EC, and the effects of temperature on leaf Rd are largely independent of the atmospheric CO_2 concentration (Gonzalez-Meler *et al*. 2004; Bruhn *et al*. 2002). The present results support these assumptions.

Temperature can also affect photosynthesis by modulating activity rates of photosynthetic enzymes and the electron transport chain (Sage & Kubien, 2007). Photosynthesis may increase as J_{ETR} increases with rising temperature; improving C gains (Lewis *et al*. 2004).Temperature effects on photosynthetic metabolism involve changes in activity of ribulose-1,5-carboxylase/oxigenase (Rubisco), as well as processes associated with the regeneration of its substrate ribulose-1,5-bisphosphate (RuBP) in the Calvin cycle (Lloyd & Farquhar, 2008). The maximum rate of RuBP regeneration, usually considered to be limited by the J_{ETR}, is generally more sensitive to temperature than RuBP carboxylation capacity, although its temperature sensitivity may also vary with growth conditions and/or genotype (June *et al*. 2004).

Photosynthesis can be limited by the export and utilization of triose phosphatesderived from the Calvin cycle. This limiting process for TPU commonly occurs at high CO_2, low O_2, high irradiance, and/or low temperatures. In cases where sugar phosphates are produced at rates higher than they are consumed, the pool of inorganic phosphate within the chloroplast may become depleted to a point of limiting photophosphorylation (Caemmerer *et al*. 2009).

In the present study, TPU was apparently not limited by EC, although an increase in TPU was observed in EC+ET (Figure 1C). The higher Rubisco carboxylation rates and maximum rate of electron transport of plants under EC+ET and ET were consistent with higher photosynthetic rates seen in these same treatments (Table 2, and Figure 1).

Chlorophyll fluorescence

Photosynthetic parameters deduced from analysis by the JIP test of transient fluorescence revealed significant differences among treatments mostly in parameters relative to absorbed radiation performance index (Pi_{abs}) and total performance index (Pi_{total}). Higher values of the performance indexes Pi_{abs} and Pi_{total} were observed in ET and EC+ET treatments for both times sampled, 7 and 14d (Table 3). The ratios Fv/Fm, Fv/Fo and Fo/Fm, used to describe the performance of the light reactions, were not sufficiently sensitive to detect changes in response to the treatments. The elevated temperature decreased total chlorophyll concentration and Chl a/b ratio, an effect which was reversed by the combination of high temperature and high CO_2 (Figure 2). In *Pinus sylvestris* chlorophyll concentration and Chl a/b ratio were also decreased by high temperature (Sallas *et al*. 2003). Elevated temperatures, even without water limitation, can result in increased oxidative stress that may affect chlorophyll and other metabolite pools (Miller *et al*. 2008). Nonetheless, based on Pi_{abs} and Pi_{total} indexes, growth and photosynthetic data, it appears that the damage caused by high temperature was not sufficient to compromise the overall physiological performance of the plants. The counteractive effect of high CO_2 under high temperature that abolished chlorophyll damage is an interesting indicator of potential compensation phenomena in predicted future climate scenarios, at least for this tree hybrid. In contrast, high CO_2 did not mitigate the harmful effects of high temperature in *E. radiata* over a long period of cultivation (150d) (Duan *et al*. 2014).

Metabolite analyses

Starch content decreased in the ET and EC+ET treatments (Figure 3A). EC promoted an increase in total hexoses, 6.48 times compared to the control (Figure 3B). This increase in soluble carbohydrates may be a result of the lower transpiration rates and stomatal conductance recorded at 14d in EC (Table 2). Increased carbohydrates accumulation in leaves may result in feedback inhibition of photosynthesis (Sallas *et al.*2003). In contrast, *E. radiata* seedlings exhibited significantly higher accumulation of starch in leaves under high temperature with minor increases upon high CO_2 after approximately 200 d of treatment exposure (Duan *et al.* 2014). These contrasting results may reflect a variation due to experimental time scale and

species characteristics. On the other hand, mature leaves of wheat grown at elevated CO_2 showed higher concentration of carbohydrates than controls, notably fructose and glucose (Drake *et al*. 1999), which is similar to the data gathered in this work. Unlike the effects of elevated CO_2, higher temperature shave been reported to cause increased respiration rates and decreased carbohydrate concentrations (Morison & Lawlor, 1999). Higher temperatures have also yielded lower leaf carbohydrates, but dark respiration rates were not changed in eucalypt hybrid (Figure 3D; Figure 3A and B).

The content of total soluble protein decreased in ET and ET+EC, whereas EC and control treatments had higher content and were not different from each other (Figure 3 C). In *Pinus sylvestris,* higher temperatures induced decreases in Rubisco, chlorophyll and soluble proteins (Sallas *et al*. 2003). For the eucalypt hybrid, however, significant reductions in chlorophyll content were restricted to ET (Figure 2). The compensatory effect of elevated CO_2 under high temperature to ameliorate chlorophyll damage maybe the result of carbon allocation to antioxidant systems, such as terpenes and other secondary metabolites capable of mitigating oxidative stress under heat (Sallas *et al.* 2003). The total protein decrease in leaves of plants exposed to ET and EC+ET treatments may be explained by a possible higher demand for nitrogen by growing parts of the plant and relocation to other organs due to the higher growth and larger leaf numbers observed in these treatments, as suggested for *Pinus sylvestris* (Sallas *et al*. 2003).

We found no significant effect of elevated [CO_2] and temperature treatments on total phenolics (Figure 3D). Likewise, in *Pinus sylvestris*, elevated CO_2 or high temperature, alone or combined, did not affect total phenolic content (Sallas *et al.* 2003). However, high [CO_2] tended to decrease total phenolics in hybrid *E. deglupta* x *E. camadulensis* (Novriyanti *et al*. 2012).

The concentration of anthocyanin was reduced by ET and EC+ET. EC promoted a slight increase in the content of anthocyanin (by 1.37 times) compared to the control, but this was not significant (Figure 3E). In C_3 plants, including most forest tree species, enriched CO_2 atmospheres increase the intracellular CO_2:O_2 ratio, thus reducing photorespiration (oxygenation) and increasing photosynthesis (carboxylation). Carbohydrates are transported throughout the plant and subsequently converted to various carbon-containing compounds required for plant metabolism, structure, storage and defense. CO_2 directly and indirectly influence carbon assimilation and nutrient acquisition and thereby the pool and fluxes of key

precursors for the production of secondary metabolites. They also may influence the signal transduction pathways that govern gene expression and subsequent synthesis of secondary compounds (Lindroth, 2010). In the high temperature treatments, anthocyanin pools may have been reduced because of a possible involvement of these metabolites as antioxidants, contributing to mitigation of oxidative stress associated with this condition (Bita & Gerats, 2013), as well as to the subsequent inhibition of their biosynthesis at persistent high temperatures (Steyn *et al.* 2002).

The activity of nitrate reductase (NR) in leaves was significantly higher in ET treatment, whereas all other treatments had equivalent NR activity (Figure 3F). In tobacco, high CO_2 caused an increase in NR activity (Geiger *et al.* 1998), which was not seen in the current study. An increase in NR activity under high temperature has been reported for barley leaves (Maevskaya *et al.* 2003), which is in accordance with the data reported herein. Since the chlorophyll precursor 5-aminolevulinic acid (5-ALA) has been shown to stimulate *Nar1* gene expression, NR protein accumulation and NR activity (Beyzaei *et al.* 2014), it is possible that lower chlorophyll content in plants under ET may have stimulated NR activity in the same treatment due to 5-ALA availability (Figure 2 and Figure 3F).

CONCLUSION

Under nitrate as a sole or major source of nitrogen and water sufficiency, higher CO_2 does not result in increased growth or improved photosynthetic assimilation. Increased temperature and its combination with high CO_2, allowed a more efficient use of available carbon, presumably due to a higher availability of reducing power made possible by photorespiration, and a consequent recovery of nitrate assimilation. Under no restriction of water and with nitrate as N source, the commercial hybrid clone of eucalypt investigated (*Eucalyptus grandis* X *E. urophylla*) performed better under high temperature, indicating the possibility of a compensatory response of high temperature to overcome the difficulties of nitrate assimilation, particularly under high CO_2 in predicted future climatic scenarios.

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FIGURES

Table 1. Effects of elevated [CO_2] (EC), elevated temperature (ET) on leaf number (plant^{-1}), plant height (cm), relative growth rate (RGR) of fresh (FM) and dry mass (DM) of shoot and root

Treatment	Leaf number*	Plantheight	RGR			
			FM shoot	FM root	DM shoot	DM root
Control	1.4 ± 0.08 c	1.20 ± 0.03 b	1.04 ± 0.19 b	0.83 ± 0.22 a	1.37 ± 0.17 a	0.72 ± 0.16 a
EC	1.7 ± 0.17 c	1.25 ± 0.05 b	1.26 ± 0.07 b	1.10 ± 0.1 a	1.32 ± 0.08 a	1.52 ± 0.12 a
ET	10.2 ± 0.76 a	1.51 ± 0.07 a	2.7 ± 0.24 a	1.74 ± 0.5 a	1.95 ± 0.2 a	1.04 ± 0.32 a
EC+ET	6.2 ± 0.53 b	1.33 ± 0.05 ab	2.36 ± 0.1 a	1.15 ± 0.07 a	1.81 ± 0.1 a	1.16 ± 0.08 a

Different letters indicate significant difference by Tukey's test ($P \leq 0.05$) or * Dunnett C test ($P \leq 0.05$). Means ± standard error of 10 replications.

Table 2. Effects of elevated [CO2] and temperature on the net photosynthetic rate (A, µmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^2 s^{-1}), transpiration rate (E, mol m^{-2} s^{-1}) and water use efficiency (WUE=A/g_s, mmol mol^{-1}), instantaneous transpiratory efficiency (A/E, µmol mol^{-1}). The values of the results refer to the relationship of the7th day on day 0 and 14th on day 0.

Treatment	7th day/day 0					14th day/day 0				
	A	g_s*	*E**	WUE*	*A/E*	*A*	g_s	*E*	WUE*	*A/E**
Control	0.89 ± 0.06 c	0.78 ± 0.04 b	0.72 ± 0.03 c	1.17 ± 0.06 a	1.24 ± 0,06 a	0.76± 0.05 b	2.60 ± 0.19 a	1.52 ± 0.07 a	0.32 ± 0.02 b	0.51 ± 0.04 b
EC	1.15 ± 0.09 bc	1.02 ± 0.17 b	1.09 ± 0.12b	1.44 ± 0.21 a	1 ±0.07 b	0.98 ± 0.07 ab	1.18 ± 0.15 b	1.13 ± 0.09 b	1.24 ± 0.18 a	1 ± 0.09 a
ET	1.26 ± 0.08 b	1.68 ± 0.14 a	1.51 ± 0.07 a	0.83 ± 0.06 b	0.86 ± 0,05 b	1.16 ± 0.11 a	1.70 ± 0.17 b	1.48 ± 0.09 a	0.87 ± 0.13 a	0.59 ± 0.05 b
EC+ET	1.60 ± 0.08a	1.97 ± 0.21 a	1.66 ± 0.11 a	1.02 ± 0.08 ab	1.02 ± 0.05 b	1.23 ± 0.05 a	1.66 ± 0.2 b	1,49 ± 0.11 a	1.08 ± 0.16 a	0.84 ± 0.04 a

Different letters indicate significant difference by Tukey's test ($P \leq 0.05$) or * Dunnett C test ($P \leq 0.05$).Means ± standard error of 30 replications.

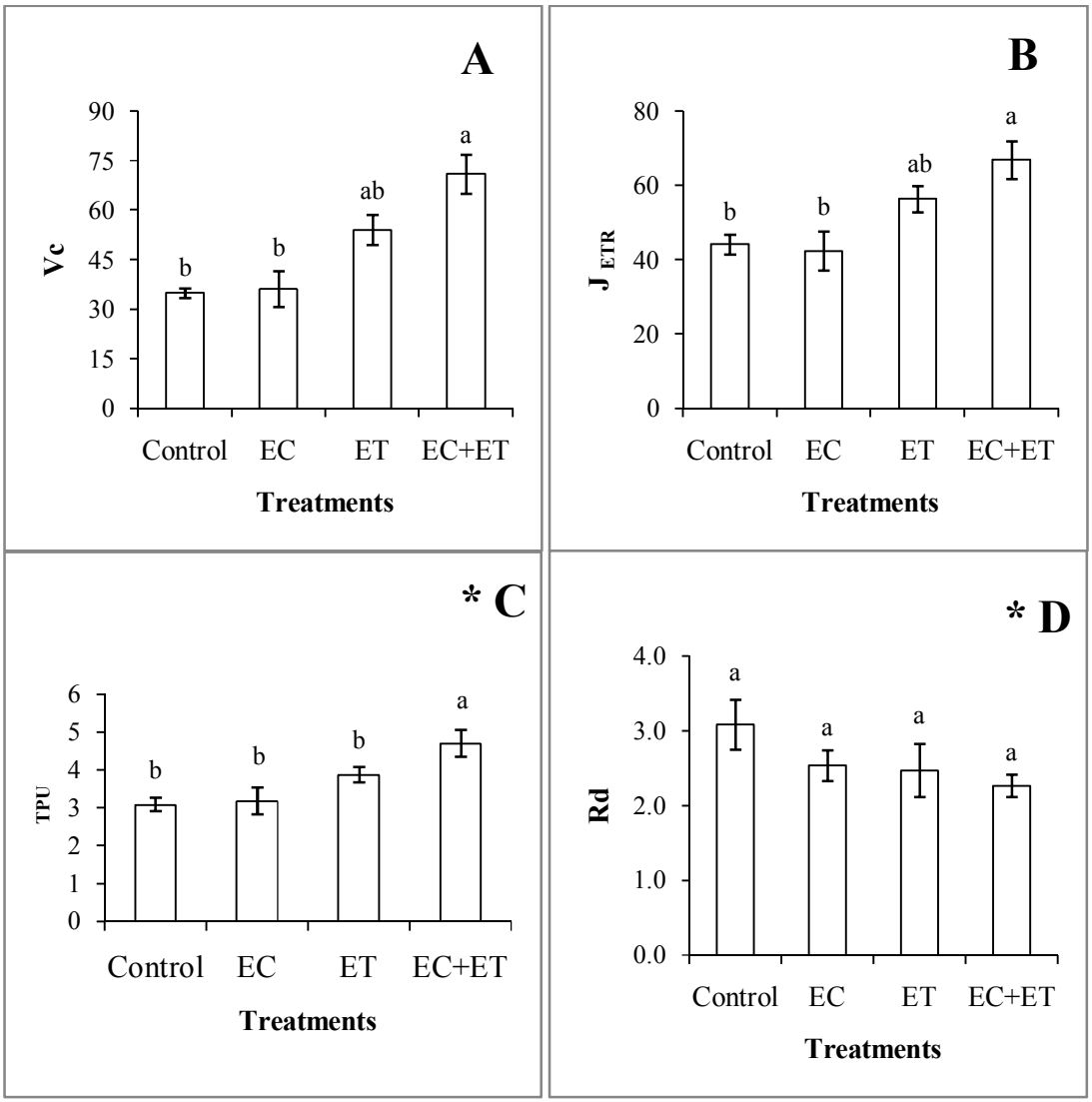


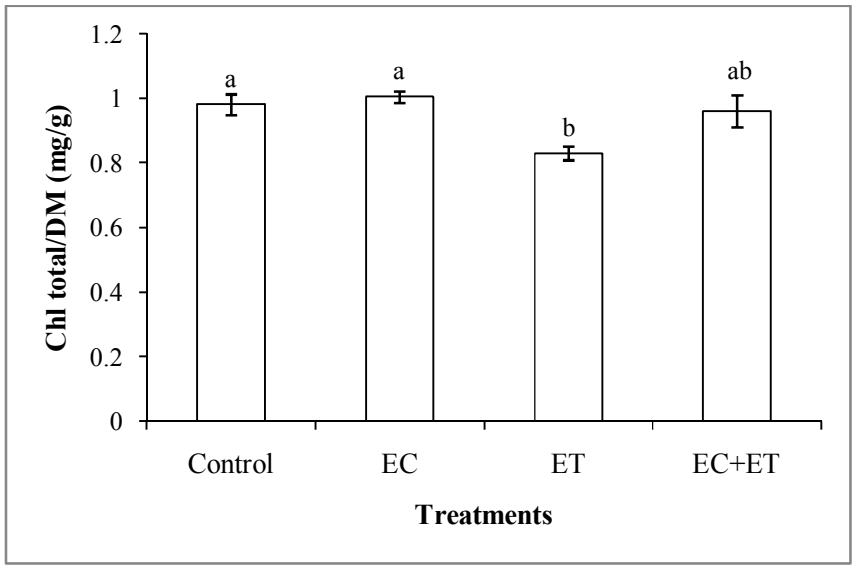
Figure 1. Effects of elevated [CO2] (EC) an temperature (ET) on the (A) Vc ‗ Rubisco carboxylation rate (μmol m^{-2} s^{-1}); (B) J_{ETR} – maximum rate of electron transport (μmol m^{-2} s^{-1}); (C) $_{TPU}$ –triose-phosphate utilization (μmol m^{-2} s^{-1}); (D) Rd - leaf dark respiration (μmol m^{-2} s^{-1}); (E). Different letters indicate significant difference by Tukey's test ($P \leq 0.05$) or * Dunnett C test ($P \leq 0.05$). Means ± standard error of 5 replications.

Table 3. Photosynthetic parameters deduced from analysis by JIP test of transient fluorescence eucalyptus plants exposed to treatments of elevated [CO2] (EC) and temperature (ET).

The values of the results refer to the relationship of the 7^{th} on day 0 and 14^{th} on day 0 for green leaves. Fv/Fm: maximum photochemical efficiency of photosystem II (PSII); Fv/Fo: ratio of variable to minimal chlorophyll fluorescence; Fo/Fm: quantum yield of dissipation; Pi_{abs}: performance index; Pi_{total}: total performance index.

	Parameters	Control	EC	ET	EC+ET
7^{th} day/day 0	Fv/Fm	1.00± 0.006 a	1.00 ± 0.01 a	1.02 ± 0.006 a	1.03 ± 0.004 a
	Fv/Fo	0.56 ± 0.04 b	1.02 ± 0.06 a	1.14 ± 0.03 a	1.15 ± 0.02 a
	Fo/Fm	0.99 ± 0.02 ab	1.01 ± 0.04 a	0.90 ± 0.02 b	0.90 ± 0.01 b
	Pi_{abs} *	0.99 ± 0.08 b	1.09 ± 0.16 b	3.55 ± 0.47 a	2.79 ± 0.22 a
	Pi_{total} *	0.78 ± 0.05 b	0.84 ± 0.15 b	3.75 ± 0.57 a	2.55 ± 0.21 a
14^{th} day/day 0	Fv/Fm	0.97 ± 0.01 a	0.97 ± 0.02 a	1.01 ± 0.02 a	1 ± 0.01 a
	Fv/Fo	0.89 ± 0.04 a	0.91 ± 0.09 a	1.04 ± 0.04 a	1.02 ± 0.05 a
	Fo/Fm	1.10 ± 0.04 a	1.14 ± 0.08 a	0.97 ± 0.03 a	1.02 ± 0.04 a
	Pi_{abs} *	0.70 ± 0.1 b	0.69 ± 0.17 b	3.06 ± 0.62 a	2.26 ± 0.37 a
	Pi_{total}*	0.41 ± 0.07 b	0.55 ± 0.14 b	2.7 ± 0.6 a	1.96 ± 0.33 a

Different letters indicate significant difference by Tukey's test ($P \leq 0.05$) or * Dunnett C test ($P \leq 0.05$). Means ± standard error of 10 replications.



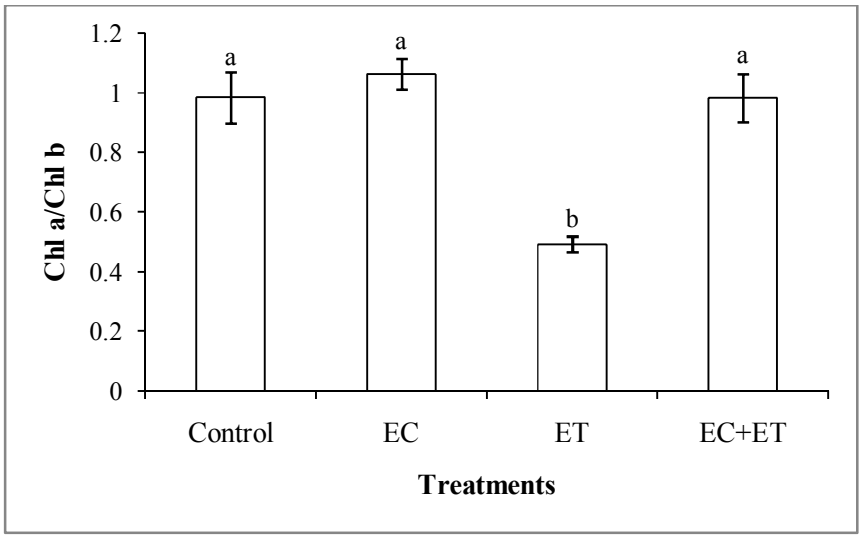


Figure 2.Effects of elevated [CO_2] and temperature on the total chlorophyll concentration (mg g^{-1} dry mass) and Chl a/ b ratio. Different letters indicate significant difference by Tukey test ($P \leq 0.05$). Means $\pm$ standard error of 4 replications.

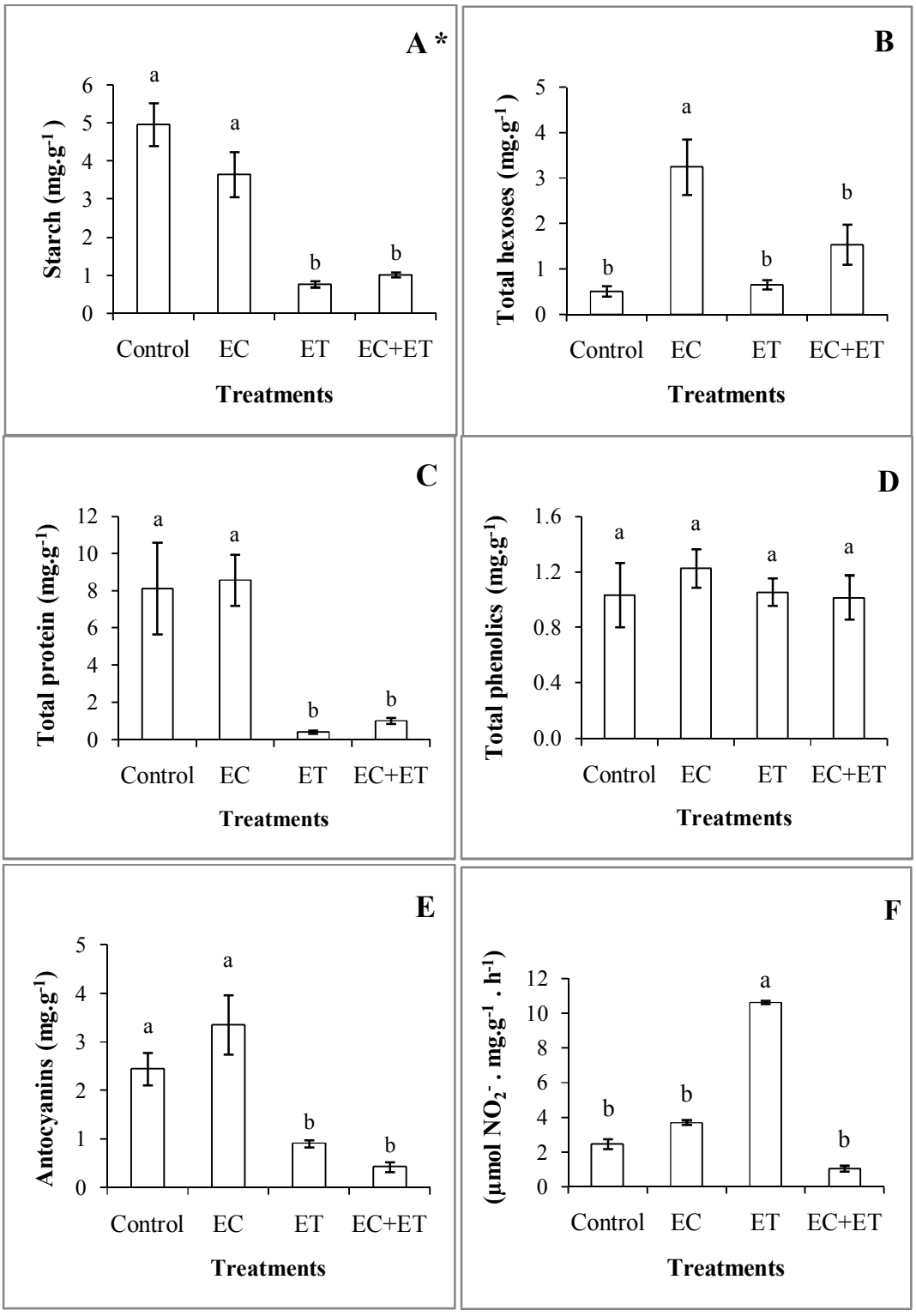


Figure 3. Effects of elevated [CO_2] and temperature on *starch ($mg.g^{-1}$) (A); total hexoses ($mg.g^{-1}$) (B); total protein ($mg.g^{-1}$) (C); total phenolics ($mg.g^{-1}$) (D); anthocyanins ($mg.g^{-1}$) (E) and

nitrate reductase activity (moles NO_2^{-}·g^{-1}edw. h^{-1}) (F) in *Eucalyptus urograndis* plants after 14 days of growth under the treatments. Different letters indicate significant difference by Tukey test ($P \leq 0.05$) or * Dunnett C test ($P \leq 0.05$).

SUPPORTING INFORMATION

Table S1. Composition of the nutrient solution

Nutrient composition	Concentration ($mg.L^{-1}$)
calcium nitrate	1250
potassium nitrate	5
potassium chloride	58
potassium sulfate	5
potassium phosphate	70
magnesium sulfate	50
iron chelate	41
boric acid	5
manganese sulfate	4
copper sulfate	0.4
zinc sulfate	0.8

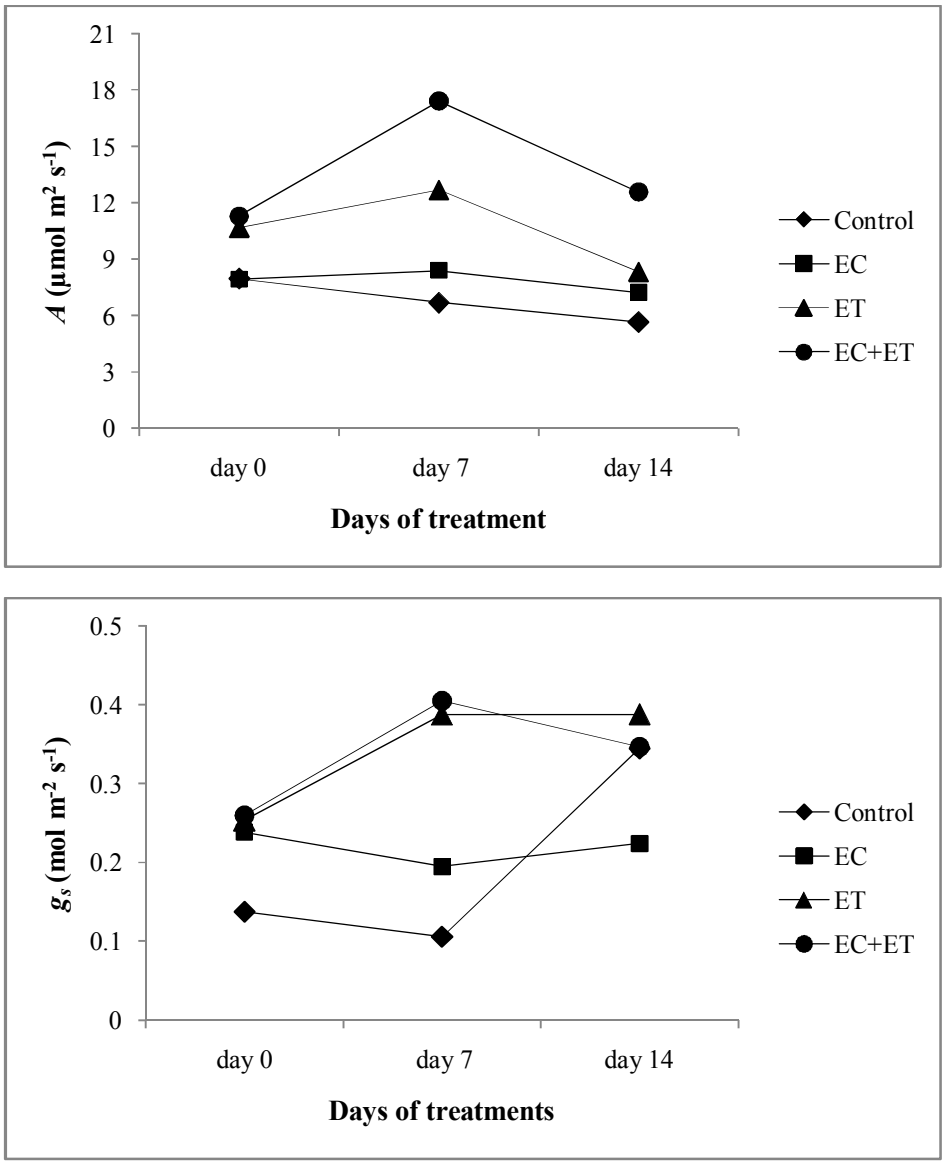


Figure S1. Effects of elevated [CO2] and temperature on the net photosynthetic rate (A, µmol m^{-2} s^{-1}) and stomatal conductance (g_s, mol m^2 s^{-1}) over time.

Considerações Finais

CONSIDERAÇÕES FINAIS

Os indivíduos jovens das espécies arbóreas estudadas nesta tese apresentaram respostas adaptativas aos estresses aplicados, envolvendo ajustes metabólicos que variaram de acordo com a situação imposta e na sua cinética de resposta. De maneira geral, os dados apontam para validade do uso de indivíduos jovens para estudos de estresse em condições controladas, de dimensões laboratoriais ou de casa de vegetação. No entanto, é necessária cautela nas tentativas de extrapolação dos resultados e padrões de resposta obtidos nestes sistemas para situações em campo, onde geralmente os estresses ocorrem de forma combinada.Além disso, determinadas respostas fisiológicas podem ser influenciadas de forma importante pela ontogenia e idade da planta. Posto isso, a seguir são tecidas algumas considerações sobre os padrões de resposta obtidos frente aos estresses aplicados e suas potenciais implicações para maior escala e envolvendo plantas mais velhas, tanto juvenis quanto adultas.

Contrariando a hipótese inicial, verificou-se que plantas jovens de *Araucaria angustifolia* apresentam características regulatórias de exsudação de resina muito semelhantes àquelas encontradas em outras espécies de coníferasevolutivamente mais derivadas, como Pinus. Mesmo os dois gêneros apresentando diferenças bastante relevantes, tais como a presença de canais de resina somente na casca em *A. angustifolia*, enquanto em *Pinus*, os canais resiníferos estão presentes na casca e no cerne; todos os eliciadores químicos testados (comumente utilizados em Pinaceae, até mesmo em operações comerciais) promoveram a exsudação de resina, inclusive com perfis cinéticos parecidos. Por exemplo, o maior tempo de exsudação foi verificado com Etrel® (gerador de etileno) e jasmonato, tal como conhecido para *Pinus elliotii.*

Considerando a distância evolutiva entre estes gêneros, isto é algo surpreendente, pois sugere que a regulação da produção de resina evoluiu relativamente cedo na história das coníferas, talvez antes da separação geográfica e especiação das coníferas da América do Norte e Sul. No entanto, também é possível que o controle da exsudação da resina possa ser o resultado de eventos independentes que convergiram para um padrão de regulação comparável entre as duas famílias de coníferas que se destacam na capacidade de resinose (Pinaceae e Araucariaceae).

Um aspecto distinto da resinose de araucária em comparação ao mesmo processo *em Pinus elliottii* é a alteração da composição de terpenos na resina eliciada por estimuladores, versus a

resina induzida apenas por ferimento. Estudos com *P. elliottii* não revelaram alterações expressivas de composição de resina estimulada, o que indica, para araucária, uma estratégia adicional de modular-se a composição de resina na direção de produtos de maior valor industrial. Porém, é necessário avaliar se este perfil se mantém em plantas de araucária mais velhas e de maior porte.

Outro ponto relevante deste trabalho foi a constatação, pela primeira vez, do efeito do óxido nítrico na produção de resina. Nossos resultados mostraram que o nitroprussiato de sódio (SNP), um doador de óxido nítrico, aumenta o rendimento da resina em mais de cinco vezes comparado com o tratamento controle. Com este resultado, abre-se um caminho de investigação do efeito de SNP na resinose de *Pinus elliottii*, por exemplo, atualmente a espécie mais explorada para resina no sul do Brasil e em diversas outras partes do mundo.

Estes resultados são bastante importantes, pois a resina pode representar uma relevante fonte de terpenos para a indústria química, farmacêutica, de alimentos e biocombustíveis. Desta forma, a extração de resina de A. angustifólia com métodos modernos de estimulação e produção pode representar uma alternativa viável para retornos econômicos a curto e médio prazo de florestas plantadas de araucária. Esta atividade poderia contribuir para promover plantios comerciais desta espécie nativa e seu uso sustentável em programas de reflorestamento.Claro está que são necessários estudos adicionais em campo e com plantas mais velhas para verificar esta possibilidade.

Uma das questões centrais associadas às previsões de mudanças climáticas é a resposta que as plantas apresentarão à elevação dos níveis de gás carbônico, aumento médio de temperatura e, em algumas regiões do planeta, restrição hídrica. Como para o sul da América do Sul e do Brasil os modelos climáticos mais aceitos predizem um aumento no regime de chuvas entra 10 e 20 %, neste trabalho optou-se por não agregar estresse hídrico como elemento experimental. No caso de espécies florestais, esta temática é revestida do uso potencial das florestas como sequestradoras de carbono em polímeros de madeira, principalmente, constituindo uma estratégia adicional de mitigação do excesso de carbono atmosférico, que causa o aquecimento do planeta por efeito estufa. O eucalipto é uma das espécies florestais de mais rápido crescimento, constituindo, no Brasil, a base da indústria de papel e celulose, principalmente na forma de híbridos interespecíficos propagados por miniestaquia em minijardins clonais.

Há inúmeras variáveis e controvérsias em relação aos efeitos das mudanças climáticas em plantas em geral e em espécies perenes lenhosasem particular. Sendo a vasta maioria das árvores plantas C3, uma das primeiras possibilidades aventadas para o clima futuro seria a chamada 'fertilização de carbono', segundo a qual, maiores níveis de CO2 promoveriam a atividade carboxilase da rubisco, aumentando o crescimento. Porém, sabe-se que esta situação nem sempre é possível, especialmente quando se combina a futura elevação de carbono com escassez hídrica, como previsto para o sudeste e norte do Brasil, por exemplo, o que tornaria impossível o benefício de carbono extra ao crescimento. Além disso, o aumento de temperatura que acompanha a elevação de CO2 tende a gerar aumentos nas taxas de fotorrespiração, podendo causar perdas expressivas de carbono fixado e, até mesmo, resultar em florestas clímax que atuam como fontes de carbono. Outro ponto importante é a questão da aclimatação ao carbono, fenômeno que resulta da exposição prolongada a este gás, resultando em estabilização ou redução da taxa fotossintética.

Em solos onde predomina o nitrato, uma das principais fontes de nitrogênio para as plantas, o aumento na concentração de CO2 pode levar a dificuldades na assimilação de nitrato, devido à necessidade de redução deste íon. Por outro lado, em plantas C3 a fotorrespiração pode servir como uma importante fonte de ácidos orgânicos e poder redutor e este processo é normalmente estimulado por altas temperaturas diurnas. Levantou-se então a hipótese de que a combinação de alta temperatura e elevado CO2, em suficiência hídrica e na presença de nitrato como fonte de N, poderia levar a um fenômeno compensatório, em que a redução e assimilação do nitrato seriam possíveis em função do poder redutor gerado pela fotorrespiração.

A análise conjunta dos dados obtidos para o clone híbrido comercial de *Eucalyptusgrandis* X *E. urophylla* indica que esta hipótese parece ser confirmada, ao menos em parte, uma vez que os parâmetros de taxa fotossintética, eficiência fotossintética e medidas de crescimento (pelo menos em alguns parâmetros e com visível tendência em outros) foram mais altos em elevada temperatura ou em elevada temperatura e elevado CO2, não ocorrendo respostas favoráveis pela simples elevação de concentração de carbono em relação ao controle. Além disso, embora seja necessário repetir o ensaio mais algumas vezes, a adição de nitrogênio reduzido (ureia) ao substrato de plantasmantidas em alto CO2, aparentemente levou a um melhor desempenho de fotossíntese e crescimento em relação à nutrição por nitrato nas mesmas condições, estando as fontes nitrogenadas alternativas em concentração equimolar de nitrogênio. Se de fato

esteresultado se confirmar, é possível que em condições similares de campo, em cenários de climas futuros, a penalização ao crescimento de florestas plantadas deste híbrido de eucalipto não seja tão expressiva.