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CONTROLE AMBIENTAL DA RESINOSE EM *Pinus elliottii* ENGELM. E SELEÇÃO DE INDIVÍDUOS SUPERRESINOSOS

Porto Alegre – Brasil

Abril de 2018

CONTROLE AMBIENTAL DA RESINOSE EM *Pinus elliottii* ENGELM. E SELEÇÃO DE INDIVÍDUOS SUPERRESINOSOS

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ANEXO 1

LISTA DE ABREVIATURAS

°C – graus Celsius

2,4-D – ácido 2,4-diclorofenoxiacético

AB – ácido abiético

APA – ácido acrilopimárico

BA – ácido benzoico

$BDALi_2$ – benzeno diacrilato de lítio

$Ca(OH)_2$ – hidróxido de cálcio

CEPA – ácido 2-cloroetilfosfônico

cm – centímetro (s)

CS – quitosano

Cu – cobre

DMAPP – pirofosfato de dimetilalilo

DOXP – 1-desoxi-D-xilulose-5-fosfato

FPP – farnesil-pirofosfato

g – grama (s)

GC – cromatografia gasosa

GPP – geranil-pirofosfato

GGPP – geranil-geranil-difosfato

G3P – gliceraldeído-3-fosfato

H_2 – hidrogênio

HMBPP – 1-hidroxi-2-metil-2(E)-butenil-4-difosfato

IC_{50} – concentração inibitória

IPP – isopentenil-pirofosfato

K – potássio

kg – quilograma

km – quilômetro (s)

KOH – hidróxido de potássio

kV – quilovolt (s)

LC_{50} – concentração letal média

Li_2CO_3 – carbonato de lítio

LIBs – baterias de íon-lítio

MDF – fibra de madeira de média densidade

MEP – 2-C-metil-D-eritritol-4-fosfato

mm – milímetro (s)

mM – milimolar (es)

MPa – megapascal

MVA – ácido mevalônico ou mevalonato

NAA – ácido naftalenoacético

NaOH – hidróxido de sódio

NFC – celulose nanofibrilada

NPs/ AgNPs – nanopartículas de prata

PAMMS – poli-α-metil-p-metilestireno

Pc – pressão crítica

PET – evapotranspiração potencial

PMYR – poli-mirceno

SA – ácido salicílico

T_C – temperatura crítica

WPC – compósitos plástico-madeira

ZnO – óxido de zinco

µA – microampere

µm – micrômetro (s)

RESUMO

A exsudação de resina (uma complexa mistura de mono, sesqui e diterpenos) após um ferimento é uma resposta de defesa típica em *Pinus elliottii.* Esta mistura complexa de terpenos encontra inúmeras aplicações industriais, incluindo aromas, fragrâncias, produtos farmacêuticos, tintas, adesivos, polímeros, solventes, etc. Para estimular o fluxo de resina, é aplicada uma pasta na superfície da ferida, que é repetida a cada quinze dias, expondo a interface entre o xilema secundário e o floema. A resina é sintetizada pelas células epiteliais e armazenada em estruturas secretoras (canais de resina), que são ativados pelo ferimento e pela aplicação, sobre a estria, de uma pasta estimuladora. Otimizar o rendimento da resina permite a utilização sustentada de produtos florestais de alto valor, que podem ser explorados nas plantações de *Pinus elliottii* durante vários anos ininterruptamente. Neste trabalho, foi realizada a investigação do rendimento de resina de árvores plantadas ou regeneradas do banco de sementes tratadas com diferentes adjuvantes químicos atuantes em diferentes mecanismos (cofatores metálicos de monoterpeno sintases, ácido benzoico e reguladores de crescimento de plantas), isolados ou em combinação, visando avaliar a existência de interações entre os mesmos e minimizar a necessidade de precursores de etileno (ethrel) na pasta indutora. A pasta com ethrel estimulou produção de resina em todas as estações. As pastas baseadas em ácido benzoico também tiveram essa capacidade, especialmente na primavera e no verão. Florestas plantadas ou regeneradas tiveram a mesma produção de resina. Foi também validado um método simples de avaliação de capacidade de resinose baseado na cinética volumétrica de liberação de resina em um período de 4h após punção do tronco para identificação de indivíduos elite para resinagem. Em paralelo, análises químicas de terebintina revelaram maiores razões de beta/alfa pineno em árvores de elevada produção de resina. Além disso, a estrutura dos canais resiníferos foi investigada por varreduras de microtomografia de raio-X em árvores de fenótipos de produção de resina distintos. As análises de estrutura de canais mostraram que o número e diâmetro de canais secretores, bem como a ocorrência de canais anastomosados foram significativamente maiores nas árvores de alto rendimento, indicando expressiva correlação entre aspectos anatômicos dos canais com o fenótipo de resinose.

Palavras-chave: *Pinus*, estação, ethrel, ácido benzoico, canais de resina.

ABSTRACT

Resin exudation (a complex mixture of mono, sesqui and diterpenes) after mechanical injury is a typical defense response in *Pinus elliottii*. This complex blend of terpenes finds numerous industrial applications including flavorings, fragrances, pharmaceuticals, paints, inks, adhesives, polymers and solvents, etc. To stimulate resin flow, a paste is applied on the fresh surface of the inflicted wound (bark streaking), which is repeated on a fortnight basis, exposing the interface between secondary xylem and phloem. Resin is synthesized by epithelial cells and accumulated in specialized secretory structures (resin canals,) which are activated by the wound and by the stimulator paste application. Optimizing resin yield allows the sustained exploitation of high value forest products, which can be produced in plantations of *Pinus elliottii* for several years uninterruptedly. Herein resin yield from planted or seed-bank regenerated trees treated with different chemical adjuvants that act in different pathways (metal cofactors of monoterpene synthases, benzoic acid and plant growth regulators), alone or in combination, was analyzed, aiming at determining the existence of interactions among adjuvants and minimizing the need of ethylene precursors (ethrel) in the stimulant paste. The paste with ethrel promoted resin yield in all seasons of the year. Benzoic acid based pastes also had this capacity, particularly in Spring and Summer. Planted and regenerated forests had the same resin yield. In addition, a simple method of resinosis capacity evaluation was validated. The method was based on the kinetic-volumetric quantification of exuded resin for 4h after puncturing the trunk for identifying elite individuals for resin extraction. In parallel, chemical analyses of turpentine showed increased ratios of beta/alfa pinene in high resin yielding trees. The structure of resin canals was determined by X-ray microtomography scans in trees of distinct resin yield phenotypes. These analyses showed that number and diameter of secretory canals, as well as the occurrence of anastomosed ducts were significantly higher in the more resinous trees, indicating a correlation between canal anatomy and resinosis phenotype.

Keywords: *Pinus*, seasonal, ethrel, benzoic acid, resin canals.

INTRODUÇÃO

Pinus elliottii Engelm. é uma espécie de conífera pertencente à família Pinaceae, nativa do sudeste dos Estados Unidos, porém amplamente cultivada no Brasil, Índia e China, principalmente devido à produção de resina (Langenheim, 2003). A resina de *Pinus* é um importante produto florestal secundário não madeireiro e uma *commodity*, com alto valor no mercado mundial. A resina é empregada como matéria-prima na fabricação de tintas, borrachas sintéticas, solventes, perfumes, materiais adesivos, materiais à prova d'água, aditivos alimentares, entre outros (Ferreira and Tomazello-Filho, 2012; Rodrigues-Corrêa and Fett-Neto, 2013; Tholl, 2006).

No Brasil, existem extensas áreas plantadas de *Pinus elliottii* com objetivo da exploração comercial de resina. Porém, estas florestas não são fruto de melhoramento genético direcionado especialmente a uma maior produção de resina de alta qualidade, tendo sido melhoradas essencialmente para uso madeireiro. Paradoxalmente, o setor resinífero vem tornando-se mais atrativo que o próprio uso madeireiro. O Brasil encontra-se na posição de segundo maior produtor mundial de resina, apresentando uma produção de 167.946 toneladas na safra 2016/2017 (ARESB, 2018). No Brasil, os principais estados produtores de *Pinus* são São Paulo, Rio Grande do Sul e Minas Gerais. No sul do Brasil, *Pinus elliottii* Engelm var. elliottii é extensamente cultivado, contribuindo com cerca de 24% da resina produzida anualmente (ARESB, 2018). O preço médio de mercado da resina brasileira em anos recentes é cerca de US$ 1.000,00 a tonelada (ARESB, 2018), sendo que cada árvore rende, em média, 3,0 kg de resina ao ano.

A resina sintetizada por espécies de coníferas é constituída basicamente por terpenos, sendo uma fonte natural abundante dos mesmos. Terpenos são metabólitos secundários derivados biossinteticamente de um composto de 5 carbonos, o isopentenil-pirofosfato – IPP - (Croteau et al., 2000; Martin et al., 2002). Com cerca de 40.000 compostos, a classe de terpenoides constitui um dos exemplos mais impressionantes na evolução de produtos químicos vegetais (Bohlmann and Keeling, 2008; Tholl, 2015). Os terpenos são classificados em diferentes classes estruturais e funcionais. De acordo com o número de unidades de isopreno na sua estrutura, os terpenos podem ser classificados em hemiterpenos (1 unidade), monoterpenos (2 unidades), sesquiterpenos (3 unidades), diterpenos (4 unidades), etc. Os terpenos mais frequentemente encontrados em óleos voláteis são monoterpenos ($C_{10}H_{16}$) e sesquiterpenos ($C_{15}H_{24}$) (Bakkali et al., 2008).

As plantas usam duas vias independentes para produzir IPP e DMAPP: a via do ácido mevalônico citosólico (MVA) e a via plasmática de metileritritol-fosfato (MEP) (Fig. 1).

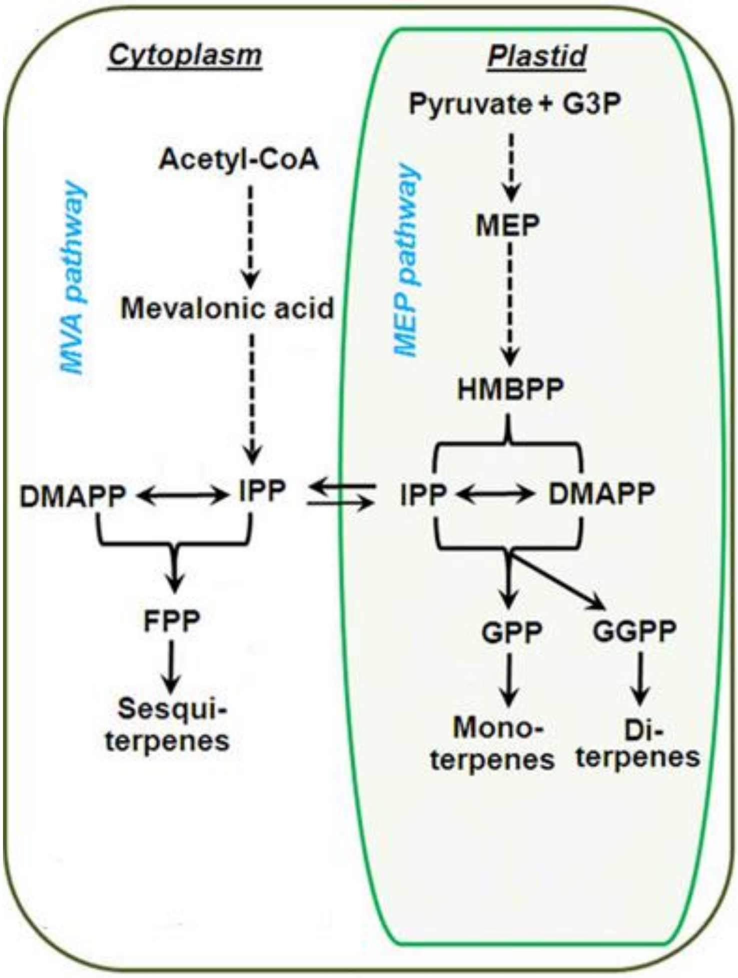

Figura 1. Biossíntese de terpenos. Adaptado de Meena et al., 2017.

Abreviações: MVA, ácido mevalônico ou mevalonato; DMAPP, pirofosfato de dimetilalilo; IPP, isopentenil-pirofosfato; FPP, farnesil-pirofosfato; MEP, 2-C-metil-D-eritritol-4-fosfato; G3P, gliceraldeído-3-fosfato; GPP, geranil-pirofosfato; GGPP, geranil-geranil-difosfato; HMBPP, 1-hidroxi-2-metil-2(*E*)-butenil-4-difosfato.

Na via do mevalonato (MVA), o IPP é formado através do ácido mevalônico, que resulta da condensação de três moléculas de acetilcoenzima-A. Na rota biossintética mevalonato independente, 2-*C*-metil-D-eritritol-4-fosfato (MEP) e 1-desoxi-D-xilulose-5-fosfato (DOXP) estão envolvidos, resultantes da condensação de piruvato e gliceraldeído-3-fosfato (G3P) (Nagegowda, 2010). A primeira via ocorre no citoplasma e leva à formação da maioria dos sesquiterpenos, enquanto a última ocorre nos cloroplastos, produzindo principalmente monoterpenos e diterpenos (Bouwmeester, 2006). IPP e DMAPP levam ao difosfato de geranilo (GPP), o precursor imediato de monoterpenos. A condensação de GPP com IPP leva ao farnesil-difosfato (FPP), o precursor imediato dos sesquiterpenos, e a condensação de FPP com IPP resulta em geranil-geranil-difosfato, o precursor de fitol, e outros diterpenos e carotenoides (De Sousa, 2015).

A resina das coníferas é composta de turpentina (ou terebintina), a fração volátil mono e sesquiterpênica, e de rosina (ou breu), a fração não volátil diterpênica (Martin et al., 2002; Phillips and Croteau, 1999; Wilbon et al., 2013), apresentando aplicações industriais distintas. Em *Pinus elliottii*, a terebintina é composta principalmente de α- e β-pineno e a rosina por ácidos abiéticos e pimáricos (Langenheim, 2003).

Ecologicamente, a síntese da resina em coníferas está diretamente relacionada ao mecanismo de defesa da planta contra insetos predadores da casca e fungos patogênicos (Fett-Neto and Rodrigues-Corrêa, 2012). A resina é sintetizada em estruturas especializadas (canais resiníferos), que são revestidas internamente com células epiteliais, dentro das quais os componentes da resina são sintetizados (Langenheim, 2003).

O lenho das coníferas (Fig. 2) é constituído por noventa por cento de traqueídes, sendo o restante composto por raios lenhosos e canais de resina (Plomion et al., 2002). Os canais de resina estão, normalmente, isolados no lenho inicial e tardio dos anéis de crescimento (Ferreira and Tomazello-Filho, 2012). Nos anéis de crescimento (Fig. 2), o lenho inicial corresponde ao incremento do tronco das árvores de *Pinus* no período vegetativo, com os traqueídes apresentando parede fina e lume amplo (coloração clara e baixa densidade). O lenho tardio é formado no final do período vegetativo, os traqueídes têm parede espessa e lume reduzido (coloração mais escura e alta densidade) (Ferreira, 2009).

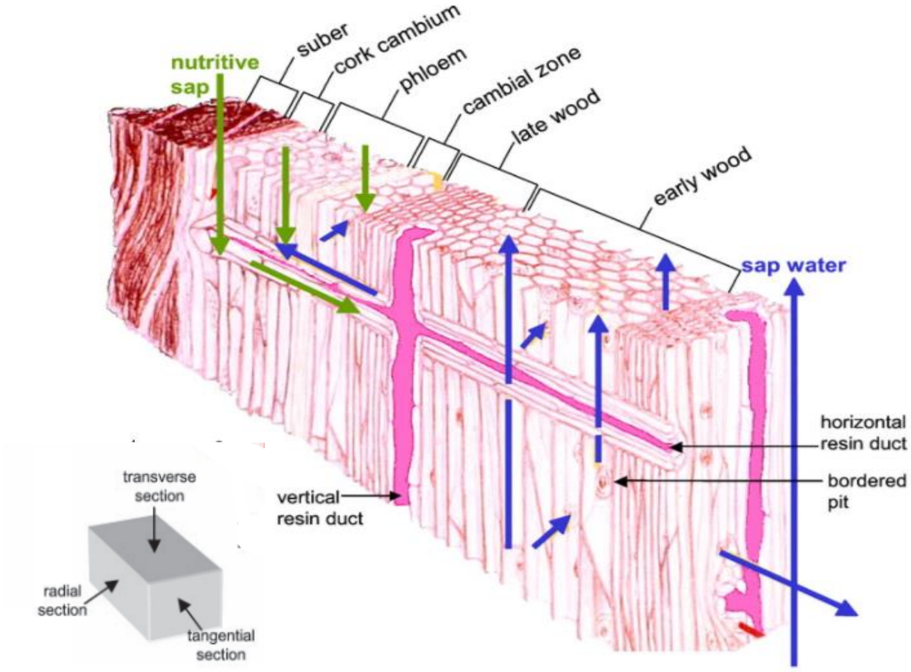

Figura 2. Esquema tridimensional da madeira de *Pinus pinaster* mostrando a estrutura relativamente homogênea do xilema de coníferas (Plomion et al., 2002).

Os canais resiníferos ocorrem no xilema secundário de *Pinus* e são horizontais (dispostos radialmente) e verticais ou axiais (Langenheim, 2003). Segundo Fahn (1982), os canais de resina axiais podem estar interconectados com os canais de resina de cada plano radial, formando uma complexa estrutura em rede.

A composição e a quantidade da resina podem ser controladas por fatores genéticos (Lombardero et al., 2000) e ambientais, incluindo irradiância (Martin et al., 2003; Peñuelas and Llusià, 1999), temperatura, índice e frequência pluviométrica, estação, incêndios florestais, fertilidade do solo, além do método e duração de extração em florestas exploradas comercialmente, enquanto os principais aspectos fenotípicos são diâmetro do tronco e tamanho da copa da árvore (Blanche et al., 1992; Cannac et al., 2009; Coppen and Hone, 1995; Hood et al., 2015; Knebel et al., 2008; Lombardero et al., 2006; Moreira et al., 2015; Neis et al., 2018; Pio and Valente, 1998; Ruel et al., 1998). Além disso, diferentes árvores dentro de populações de uma mesma espécie podem variar quimicamente, bem como ao longo das estações em resposta ao estresse ambiental e em função da idade (Brito et al., 1978; Katoh and Croteau, 1998; Lombardero et al., 2000; Peñuelas and Llusià, 1999).

Embora seja reconhecido que todas as espécies de *Pinus* são capazes de sintetizar constitutivamente resina em maior ou menor quantidade, sabe-se que a produção também pode ser induzida e modulada quimicamente com a aplicação de pastas estimulantes destinadas para esse fim (Fig. 3). Assim, pesquisas para otimizar a produção de resina induzida têm sido realizadas, a partir da modificação da pasta base comercialmente utilizada, a qual é composta basicamente de ácido sulfúrico e CEPA (ácido 2-cloroetilfosfônico, um precursor sintético de etileno) (Fett-Neto and Rodrigues-Corrêa, 2012), os principais componentes ativos que atuam como indutores da oleorresinose.

Figura 3. Aplicação da pasta na estria feita no tronco.

Dentre os vários estudos realizados para testar as respostas de diferentes vias de sinalização no metabolismo vegetal estão: o uso de diversos indutores como paraquat (gerador de radicais livres), extrato de levedura (mimetizando ataque por patógenos), ácido salicílico (participa da sinalização de resposta ao ataque por patógenos), auxina (induz a formação endógena de etileno; estimula a diferenciação de dutos resiníferos), ácido jasmônico (sinalizador de dano mecânico e herbivoria; indutor de diferenciação de dutos resiníferos) e íons metálicos (potássio, ferro e manganês, que são cofatores das terpeno-sintases de coníferas) (Martin et al., 2002; Rodrigues et al., 2011; Rodrigues et al., 2008; Rodrigues and Fett-Neto, 2009). No entanto, as florestas comerciais manejadas ainda comportam uma capacidade limitada de produção de resina. Uma das razões seria a limitação da expansão da área comercial. Outros fatores residem na falta de genótipos uniformes e altamente produtores de resina e no fato de que os programas de melhoramento necessitam de muitos anos para a seleção e geração de árvores-elite.

Objetivos

Tendo em vista o alto valor de mercado atribuído à resina e seus derivados (breu e terebintina), bem como a importância sócio-econômica-ambiental desta atividade no litoral do RS, especialmente em função da intensificação da atividade florestal no estado, os objetivos deste trabalho foram:

- Avaliar o rendimento de resina de árvores tratadas com diferentes adjuvantes químicos isoladamente ou em combinação, visando substituir ou reduzir a necessidade de precursores de etileno.
- Comparar a produção de resina em florestas plantadas versus regeneradas a partir do banco de sementes, seguido de manejo mecânico.
- Identificar marcadores químicos de composição de resina que estejam correlacionados com produtividade de biomassa de resina e selecionar, por meio do uso desses marcadores químicos, genótipos altamente produtores de resina de *Pinus elliottii*.
- Avaliar e selecionar genótipos com alto rendimento de resina usando análise cinética-volumétrica.
- Investigar a existência de correlação entre número, forma, área e volume interno de canais resiníferos e a produção de resina.

Hipóteses

1) O uso de adjuvantes de pasta indutora que atuam em vias diferentes de sinalização tem efeito sinérgico na produção de resina;
2) Florestas plantadas e aquelas regeneradas a partir do banco de sementes seguido de manejo mecânico possuem produções de resina equivalentes.
3) A seleção de indivíduos de maior capacidade resinífera pode ser feita por análise química de marcadores de terebintina e/ou por método de cinética-volumétrica de liberação de resina após punção, seguida de avaliação passado um período de poucas horas.
4) Árvores superresinosas possuem maior número de dutos resiníferos e maior lúmen de dutos resiníferos.

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CONTEÚDOS ABORDADOS

Os resultados obtidos durante o doutorado estão organizados nesta tese em capítulos e anexos, na forma de artigos científicos publicados ou a serem submetidos à publicação.

O **Capítulo 1** apresenta manuscrito a ser submetido ao periódico *Industrial Crops and Products*. Trata-se de uma revisão de literatura acerca das atuais aplicações de produtos florestais não madeireiros oriundos de pinheiros.

O **Capítulo 2** apresenta manuscrito a ser submetido ao periódico *Frontiers in Plant Science.* Este capítulo descreve um novo método de identificação precoce de genótipos com alto rendimento de resina e faz uma análise comparativa dos canais resiníferos das árvores com alta e baixa produção de resina.

O **Anexo 1** foi publicado no periódico *Industrial Crops and Products*. Este capítulo trata da aplicação de novas pastas estimulantes de resina e compara floresta plantanda com floresta regenerada, em relação ao rendimento de resina.

O **Anexo 2** apresenta o número do registro de Patente, que tem como título: Método de identificação precoce de genótipo com alta produção de resina e método para estabelecer florestas resinosas compostas de genótipos superresinosos e homogêneas para resinagem.

CAPÍTULO 1

Non-wood uses of *Pinus* spp.: boundless?

Franciele A. Neis, Fernanda de Costa, Artur T. de Araújo Jr., Janette Palma Fett, Arthur G. Fett-Neto.

Artigo a ser submetido ao periódico *Industrial Crops and Products.*

Non-wood uses of *Pinus* spp.: boundless?

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Abstract

Pine trees dominate large areas of the world's forests, occupying diverse environments, successfully colonizing regions that exhibit extreme variations in temperature, photoperiod, availability of water, irradiance and soil nutrients, as well as pose challenges by different herbivores and pathogens. In part this is a result of the acquisition of a complex defense system which is based on the secretion of resin, a complex mixture of terpenes produced by specialized cells. Terpenes form resin find an array of applications in industry sectors, including chemicals, pharmaceuticals, agrochemicals, food additives, and bioenergy. More recent potential uses of resin products are biodegradable batteries and as components in the manufacturing of biodegradable plastics. Besides resin, other non-wood products from pines include needles, cones and bark, which have been used for landscaping, bioenergy, and show great potential as plant cultivation substrates, bioherbicides, ingredients in MDF, metal biosorption composites, among other applications. The availability of industrial quantities of renewable non-wood biomass is a topic of significance due to the depletion of fossil-based raw materials and the increasing awareness of the negative impact of their use on air quality and global climate. In this paper, a general overview of the current and emerging myriad applications of non-wood pine products is provided, highlighting the importance of fully exploiting these forest resources in a sustainable fashion.

Key words: Pine oleoresin applications, terpenes, non-timber forest products, pine cone, green plastic.

1. Pine resin features and main chemical uses

Various products from nature have been instrumental in allowing technological advances throughout history and for the development of different civilizations. Resin produced by conifers is one of the key examples of such products. Pine tar and pitch were used for caulking the seams of wooden ships and vessels (Coppen and Hone, 1995; Rodríguez-García et al., 2015; Snow, 1949). Pine resin was also used as the base for the embalming fluid during the mummification process (Giuffra et al., 2011). For centuries, pine-based products have been widely used in adhesives, soaps, water-repellent surface coatings for ropes and construction (Mills and White, 1977; Russo and Avino, 2012).

The evolutionary success of the conifers rests in part on the complexity of their defense mechanisms to deter herbivore and pathogen predation (Nystedt et al., 2013; Trapp and Croteau, 2001). In conifers, responses to abiotic external conditions, as well as biological factors, such as insects and pathogenic microorganisms, are modulated by the production of resin secretions in specialized wood canals (Ferreira and Tomazello-Filho, 2012), protecting and preserving the injured part of the tree (Ulukanli et al., 2014). Coniferous resin is a complex mixture of secondary metabolites (Vilanova et al., 2014). Terpenoids, flavonoids, and fatty acids are the main components of resin (Trapp and Croteau, 2001). The exuded resin of the plant is formed by turpentine (a volatile fraction, formed by mono- and sesquiterpenes) and rosin (a non-volatile fraction, formed by diterpenes) (Fig. 1) (Bohlmann and Keeling, 2008; Phillips and Croteau, 1999; Zulak and Bohlmann, 2010).

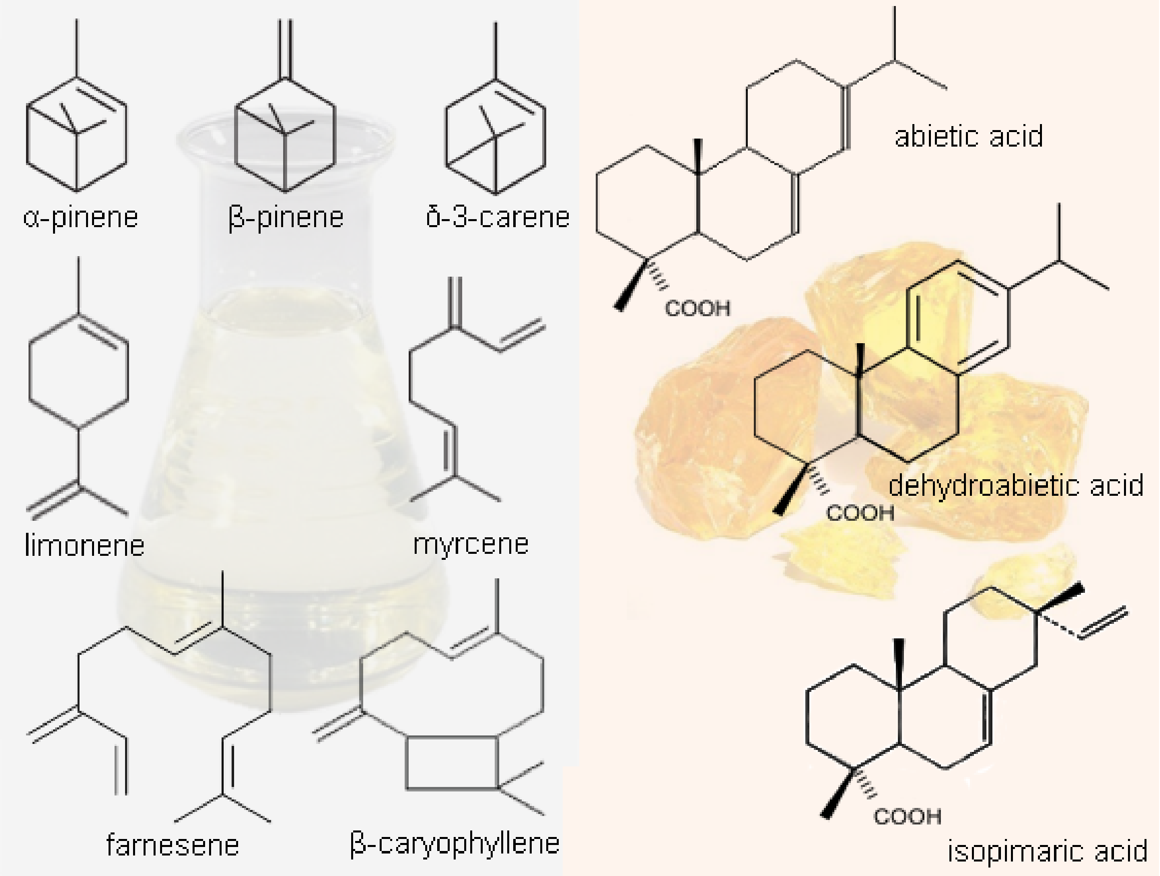

Fig. 1. Major components of pine resin. Turpentine (volatile liquid fraction, left panel) formed by monoterpenes (*e.g.* α-pinene, β-pinene, limonene, δ-3-carene and myrcene) - and sesquiterpenes (e.g. β-caryophyllene and farnesene). Rosin (non-volatile solid fraction, right panel), formed by diterpenes (*e.g.* abietic acid, dehydroabietic acid and isopimaric acid).

Rosin is one of the natural gums obtained from resin exuded by conifers that has been extensively used because of its wide range of bioactivities (Li et al., 2013; Naumov et al., 2015; Yadav et al., 2016). Rosin consists primarily of abietic- and pimaric-type resin acids (also named rosin acids) (diterpenoids containing 20 carbons) with characteristic hydrophobic hydrophenanthrene rings, which give them excellent film-forming properties (Yadav et al., 2016). Rosin is employed in the manufacture of paints, varnishes, plastics, lubricants, adhesives, asphalt, rubber, insecticides, germicides, cosmetics, chewing gums and numerous pharmaceutical products. One of its main applications, however, is in the manufacture of glue rosin with widespread use in the paper industry (Phun et al., 2017; Rodrigues-Corrêa et al., 2013; Yadav et al., 2016).

The volatile fraction of pine resin, turpentine, is made up of a mixture of terpenes - compounds that share isoprene (2-methyl-1,4-butadiene) as the common carbon skeleton building block and can therefore be classified according to the number of such units (Gandini, 2011). Mono and sesquiterpenes, containing 10 and 15 carbons, respectively, are the major constituents of volatile oils and widely used in industry as solvents for paints and varnishes. This fraction can also be used as a cleaning solvent and a starting material for pharmaceuticals and other organic compounds, fragrances, aromas in perfumes, food flavorings and in the industry of insecticides (Phillips and Croteau, 1999; Rodrigues-Corrêa et al., 2013) The main components of turpentine are unsaturated hydrocarbon monoterpenes such as α-pinene, β-pinene, and δ-3-carene (Buisman and Lange, 2016).

2. Other non-wood uses of *Pinus* spp.

2.1. Agriculture applications

2.2.1 Rosin

In agriculture, synthetic herbicides are widely used for controlling invasive plants (Lin et al., 2011). However, misguided or excessive use of these herbicides may cause harmful effects, such as toxicological and environmental impacts (Dayan and Duke, 2003), and, for this reason, considerable efforts have been undertaken to turn natural products into value-added herbicide preparations as an alternative to reducing environmental impact. In this context, acrylopimaric acid (APA) derivative compounds - prepared through a reaction between acrylic acid and the non-volatile rosin fraction exhibit high biological activity against *Amaranthus retroflexus*, *Echinochloa crus-galli*, and *Brassica campestris* L., making it a potential component for herbicide formulations, which can be applied in agriculture as an environmentally friendly weed manager (Gao et al., 2015).

2.2.2 Volatile oil from needles

Another example of compounds with potential to control pests are volatile oils conifer needles, which are known for their richness in volatiles (Amri et al., 2017). Volatile oils are obtained by hydrodistillation of needles and contain oxygenated diterpenes, sesquiterpenes and monoterpenes. Black pine (*Pinus nigra* subsp*. laricio*) volatile oil has already been shown to significantly inhibit the growth of three common weed seedlings: *Phalaris canariensis* L., *Trifolium campestre* Schreb. and *Sinapis arvensis* L. in a dose dependent manner (Amri et al., 2017). Hamrouni et al. (2015) reported allelopathic effects of volatile oils of *Pinus halepensis,* which displayed both antifungal and herbicidal activities. The amount of α-pinene in the volatile oils was directly linked to the inhibitory effect on seed germination and seedling growth.

Amri et al. (2011) observed that *Pinus patula* volatile oils also displayed inhibitory action on germination and seedling growth o*f S. arvensis, Lolium rigidum, P. canariensis and T. campestre*. Volatile oils of *Pinus pinea* were characterized by high content of monoterpenes, specially limonene (more than 50%) and also exhibited inhibitory effects on seed germination and seedling growth against common weeds (Amri et al., 2012).

2.2.3. Pine mulch (needle litter and bark)

Pine straw is a byproduct of a natural biological process, leaf loss and decay (Dyer et al., 2015). Pine straw and/or bark mulch has emerged as an useful commercial product for horticultural crops and landscaping in urban and suburban areas (Dickens et al., 2012; Duryea and Edwards, 2009). Pine mulch has several beneficial effects as soil cover; it helps soil and root insulation, moist retention, as well as prevents weeds, erosion and pathogen spread by soil splashing (Maggard et al., 2012).

Currently, fallen pine needles in pine plantations are a valuable commodity in the southeastern U.S. (Minogue et al., 2007). According to Dickens et al. (2012), pine straw revenues have helped many landowners maintain reasonable cash flows to achieve attractive rates of return on their forestland investment. In addition, pine straw harvesting is combined with timber production and other land uses to increase earnings (Dyer et al., 2015). Removal of excess biomass accumulation is also important for the prevention of forest fires (Susaeta et al., 2012). The main commercial species for the production of pine straw are slash pine (*Pinus elliottii* Engelm.), longleaf pine (*Pinus pallustris* Mill.) and loblolly pine (*Pinus taeda* L.) (Dickens et al., 2012).

A potential application for the large needle biomass stock is the use as an herbicide and/or plant growth substrate (Rodrigues-Corrêa et al., 2017). The aqueous extracts of green needles of *Pinus elliotti* were seen to have an inhibitory allelopathic activity on lettuce germination and seedling growth which directly increased with concentration and time of short term postharvest dry storage at room temperature (Rodrigues-Corrêa et al., 2017). Kato-Noguchi et al. (2017) reported the presence of the allelopathic substances 15-hydroxy-7-oxodehydroabietate and 7-oxodehydroabietic acid in Japanese red pine (*Pinus densiflora* Sieb. et Zucc.), which are formed by the degradation process of abietic acid in the soil, suggesting that allelopathy may be involved in the establishment of a sparse understory vegetation, preventing the invasion of herbaceous plants. In contrast, in the afore mentioned study with slash pine, pine litter (fallen and weathered needles of brown color) promoted growth of lettuce seedlings and proved to be a neutral growth substrate for cucumber and tomato plants, comparable to rice husk (Rodrigues-Corrêa et al., 2017). Hence, slash pine needles displayed a dual allelopathic effect, being inhibitory when harvested as green needles and stimulatory when in litter form. Pine bark has long been used in landscaping, providing a relatively neutral substrate for plants and a soil covering material.

Aqueous extracts obtained from shoots of young *Pinus halepensis* were also reported as being allelopathic and this negative effect in germination and growth of the understory impact the plant biodiversity in Mediterranean (Fernandez et al., 2013). In addition, there are reports on allelopathic activity of other *Pinus* spp., such as *Pinus halepensis*, *Pinus pinea* and *Pinus massoniana* (Alrababah et al., 2009; Hamrouni et al., 2015; Hou et al., 2012; Valera-Burgos et al., 2012).

2.2.4. Urease inhibitor

Urea is largely used as a nitrogen fertilizer in agriculture worldwide; however, losses of nitrogen as a result of the volatilization of ammonia lead to a decrease in its efficiency (Artola et al., 2011; Suescun et al., 2012). Different methods have been developed over the years to reduce these losses and improve nitrogen fertilizer formulations. One of the alternatives involves the use of urea combined with urease inhibitors derived from natural products (Modolo et al., 2015). Urease inhibitors delay the hydrolysis of urea, increasing the chances of its incorporation into the soil by rain, irrigation or mechanical operations (Artola et al., 2011).

In an attempt to increase fertilization efficiency by delaying urea hydrolysis in the soil, Kundu et al. (2013) developed a protocol for coating urea with pine resin from *Pinus roxburghii*. Coated urea contained 3-4% of pine resin and 44 % of nitrogen. Regardless of the soil type, urease activity decreased considerably when fertilization was with pine resin-treated urea when compared to control receiving conventional urea. Pine resin acted as a physical barrier around the urea granules slowing the release of nitrogen. In addition, it inhibited urease activity through its antibacterial properties, and due to its acidity, it inhibited volatilization loss by reducing alkaline microsites. As a result, pine resin appeared to be a good alternative as urease inhibitor in soil and it could be a substitute for the commercial neem-coated urea (Kundu et al., 2013; Kundu et al., 2018).

Another study evaluated several extracts from plants, such as *Acacia caven*, *Quillaja saponaria*, *Bacharis linearis* and *Pinus radiata*, with the aim of measuring their effects on nitrogen transformation, soil respiration, soil microbial biomass and in urease activity. Both *Pinus radiata* ethanolic extract from barks and aqueous extract from its leaves contained high levels of phenols and inhibited soil nitrification and mineralization. Pine extract from barks also decreased soil respiration and microbial biomass (Suescun et al., 2012).

2.2. Biofuels

The search for clean and renewable energy alternatives to replace current fossil-based sources and reduce emissions is a constant activity. Pine oil, synthesized from pine resin, either tapped or extracted from wood, twig and cone biomass, has been viewed as a potential renewable source of fuel for diesel engines (Fig. 2A). In general, pine oil is produced from resin, which is collected from the pine trunk by tapping, followed by steam distillation, which separates turpentine from rosin. Then, turpentine passes through an acid-catalyzed hydration process with ortho-phosphoric acid to synthesize different grades of pine oil (Fig. 2B) (Vallinayagam et al., 2015). The constituents of pine oil are terpineol, which is a tertiary alcohol, dipentene (an isomer of pinene), unreacted pinene and some minor quantities of other by-products and impurities (Fig. 2C) (Vallinayagam et al., 2013, 2014a).

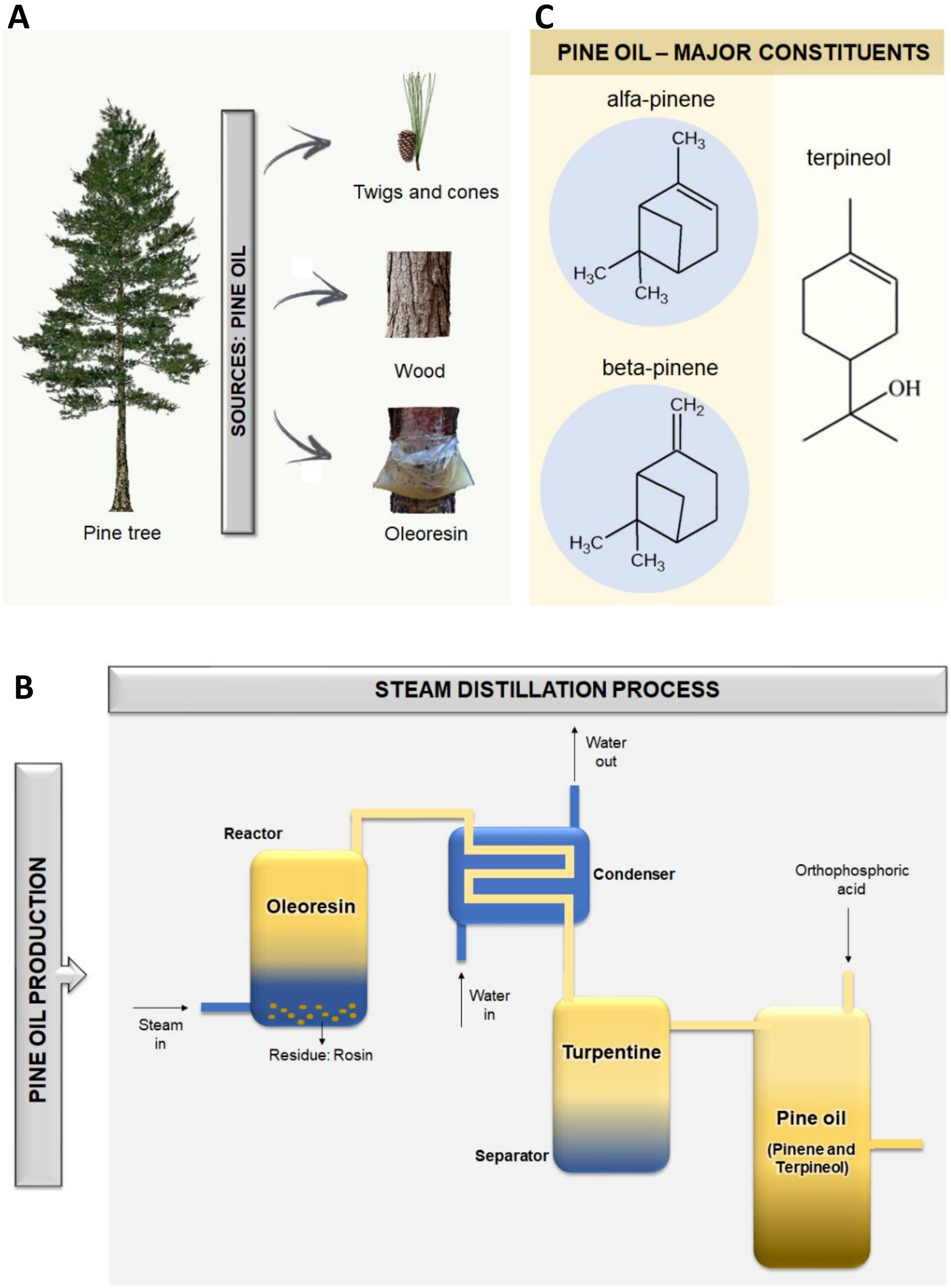

Fig. 2. Pine oil production by steam distillation. (A) sources; (B) production sequence; (C) major components of pine oil. Based on Vallinayagam et al. (2015).

As key advantages, pine oil is essentially stable under all conditions of use and storage, and highly versatile. Raw material originated from the forest can readily be mixed with petroleum diesel. In addition, the estimated thermal and physical properties of pine oil are suitable for use in diesel engines with the notable advantages of lower viscosity and boiling point and a calorific value comparable to diesel its (Vallinayagam et al., 2013, 2014a).

Vallinayagam et al. (2013) showed that the presence of pine oil biodiesel could reduce carbon monoxide, hydrocarbon, and smoke emissions by 65%, 30% and 70%, respectively, with the drawback of increasing nitrogen oxides emission compared to diesel at full load condition. It was also shown that brake thermal efficiency and maximum heat release rate, important engine parameters, increased by 5% and 27%, respectively. Similarly, Vallinayagam et al. (2014a) have evaluated the performance, combustion and emission characteristics of a double biofuel composed by kapok methyl ester (from *Ceiba pentandra*) and pine oil blend. The combined fuels could be used directly in diesel engines without any modification and their hydrocarbon, carbon monoxide and smoke emissions at full load were reduced by 8.1%, 18.9% and 12.5%, respectively. In addition, oxides of nitrogen emission remained similar to those of standard diesel.

Turpentine has been shown to efficiently replace fossil fuels. Conventional fossil fuel used in diesel engines contains higher amounts of aromatics and sulfur, which cause environmental pollution. Biofuels, on the other hand, appear to be a more environmentally friendly energy and renewable alternative. Turpentine oil can be obtained either by pyrolysis mechanism or by resin fractioning from pine trees. This volatile fluid can be used as a biofuel of lower exhaust emission properties, despite being slightly more expensive than conventional petroleum fuel (Anand et al., 2010; Vallinayagam et al., 2014b). Anand et al. (2010) evaluated the combustion performance and exhaust gas characteristics of turpentine fuel mixed with conventional diesel fuel in a diesel engine and observed lower carbon monoxide, hydrocarbon, oxides of nitrogen, smoke and particulate matter for a proportion of 30% turpentine blended with diesel.

Sulfate turpentine from kraft pulp mills can be added to pure gasoline (at 5–10% w/w), yielding improving several engine performance parameters, including mean effective pressure, thermal efficiency, specific fuel consumption, and brake power. Turpentine also decreased carbon monoxide in the exhaust of gasoline engines, albeit it increased some pollutants such as nitric oxides and unburned hydrocarbons (Yumrutaş et al., 2008).

Another renewable energy alternative is the use of lignocellulosic biomass to produce bioenergy considering its high energy potential and availability (García et al., 2017). In this direction, Moncada et al. (2016) proposed a profitable biorefinery scheme for the conversion of *Pinus patula* barks into ethanol and furfural. In addition, García et al. (2017) developed some scenarios for the production of hydrogen through the gasification and dark fermentation using *Pinus patula* raw material as energy source. The results showed that the use of thermochemical processes (e.g. gasification) in biorefining projects is attractive because of the flexibility to process a variety of biomass feedstock and produce products with a wide range of large-scale applications.

Harvey et al. (2009) suggested that α- and β-pinene are potential targets for fuel production. Energy density and heat of combustion of pinenes can be increased by dimerization under moderate conditions (100 °C and atmospheric pressure) with heterogeneous solid acid catalysts Montmorillonite K-10 (MMT - K10) and Nafion. These pinene dimers have a volumetric heating value similar to the JP-10 jet fuel and are proposed as an alternative to aviation and rocket fuels.

Velmurugan et al. (2015) described an efficient process for the enzymatic conversion of radiata pine and other potentially softwoods into a sugar syrup suitable for conversion into fuels and chemicals. In addition, the lignin from the process remained comparatively unmodified, providing an opportunity for conversion into saleable co-products.

Research on new sources of biomass using waste from corn mills in addition to raw material obtained from pine (*Pinus radiata* sawdust), showed that a mixture of 50% of corn powder combined with 50% *Pinus radiata* is a suitable material for the production of granules, so that corn powder pellets reduce the total biofuel cost (Fernández-Puratich et al., 2017).

2.3. Green batteries

Lithium-ion batteries (LIBs) are widely used, especially in portable electronic devices such as laptops, cameras and cell phones, underlining the increasing need for chemical energy storage devices. However, the standard electrode materials are made of lithium transition-metal-inorganic oxides or phosphates (such as iron, manganese, cobalt or titanium), which are non-renewable and finite resources. Thus, replacement by organic electrodes, especially if these organic materials are derived from biomass, is beneficial to the environment, favoring recyclability (Poizot and Dolhem, 2011) and reducing environmental damage. Various kinds of organic electrode materials have been exploited. Renault et al. (2013) reported preliminary potential results on dilithium *trans–trans* benzenediacrylate ($BDALi_2$), an organic material for lithium-ion batteries. The $BDALi_2$ can be produced from natural compounds and it is synthesized through condensation of malonic acid (a natural compound found in fermented fruit, fruit vinegars and alfalfa) and terephthalaldehyde (prepared through the reduction of terephthalic acid, which can be produced from limonene, α or β-pinene) (Colonna et al., 2011; Lin et al., 2011; Renault et al., 2014).

The recycling of lithium and other materials after the battery is used is also a key point in the production process of environmentally friendly batteries. It starts with the opening of the battery and separation of all components based on their solubility in H_2O or ethanol. However, $BDALi_2$ decomposition products formed during the battery closed circuit cycle create a problem. These impurities make it impossible to reapply the recycled electrode material back to the battery. Therefore, one of the alternatives is to destroy the battery thermally. Thermally decomposing the battery yields a pure powder of Li_2CO_3, a starting material for new synthesis (Renault et al., 2014).

2.4. Insecticides

Insecticides play an important role in agricultural production; however, continuous use may lead to the development of resistance in target insects (Wang et al., 2014). The insecticidal synergists make a great contribution to dealing with resistance problems in insecticide applications (Cui et al., 2017). Wang et al. (2014) showed that conifer extract fractions inhibited insect glutathione S-transferase activity using the *Leptinotarsa decemlineata* (colorado potato beetle) model. Active fractions contained both the flavonoid, taxifolin, detected in the cones from *Picea mariana* and *Pinus banksiana* and *Larix laricina* bark, and a lignan, (+)-lariciresinol 9-*p*-coumarate (Fig. 3), detected in the cones from *Picea mariana* and the bark from *Larix laricina* and *Abies balsamea*, suggesting that these compounds can be considered as potential new insecticide synergists.

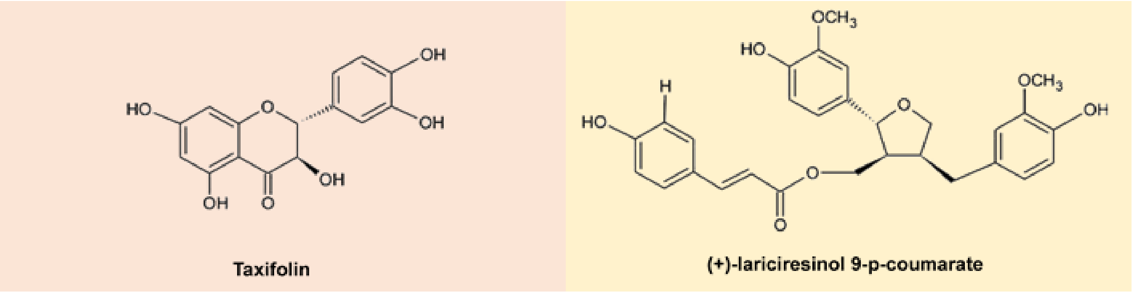

Fig. 3. Chemical structures of phytochemicals identified in conifer extracts that have insect toxicity properties.

The western pine beetle *Dendroctonus brevicomis* LeConte is a major agent of mortality of *Pinus ponderosa*. *Dendroctonus* species, including *D. brevicomis,* produce the antiaggregation pheromone verbenone, which is used as a pesticide against *D. ponderosa*, the mountain pine beetle, and *D. frontalis* Zimmermann, the southern pine beetle. Verbenone is a product of pinene oxidation and functions as an imago dispersing signal, driving beetles away from fully infested trees. By treating trees with this semiochemical, the bettle repelling signal avoids mass attacks of insects on trees. Bioassays with *Pinus ponderosa* determined that the effect of a verbenone releasing pouch was stable for a distance up to two meters from the device. The authors suggested that verbenone is best used in combination with other semiochemicals (Fettig et al., 2009). On the other hand, verbenol, the other main oxidation product of pinene, has insect aggregation properties that signal for mass attacks on trees and, therefore, may be used as an attractant to insecticide traps. Both these pinene derivatives may be produced by biotransformation methods (Limberger et al., 2007).

Volatile oils can be used as an alternative to synthetic larvicides for vector control programs (Pavela, 2015). For example, the volatile oil from fresh needles of *Pinus brutia, Pinus halepensis and Pinus stankewiczii* showed substantial larvicidal effect on mosquitoes (*Aedes albopictus*) and good insect repellent activity (Koutsaviti et al., 2015). The volatile oil from fresh needles of *Pinus kesiya* is composed mainly by β-pinene, α-pinene, myrcene and germacrene D and was tested against larval stages of the malaria vector *Anopheles stephensi*, the dengue vector *Aedes aegypti* and the filariasis vector *Culex quinquefasciatus,* Biosafety tests were performed with aquatic non-target organisms such as *Gambusia affinis*, *Diplonychus indicus* and *Anisops bouvieri* with LC_{50} values varying from 4.1 to 8.4 mg/ml. No harmful consequences were established for applications below 500 µg/ml, which led to 100% mortality of the targeted immature mosquitoes (Govindarajan et al., 2016). The difference observed in toxicity is possibly caused by the different concentration of pinenes, which are often the main active larvicide compound in conifers (Govindarajan et al., 2016; Koutsaviti et al., 2015).

Volatile oils of needles from *Pinus sylvestris* had good larvicidal toxicity against *Aedes aegypti* and *Culex quinquefasciatus* (Fayemiwo et al., 2014). Volatile oil of twigs with leaves from *Pinus nigra* has also been reported as an effective larvicide against *Culex quinquefasciatus*. Simple binary blends of volatile oils such as *Satureja montana* + *Pinus nigra* (1:1), however, displayed an antagonistic effect, leading to a higher IC_{50} (Benelli et al., 2017). Furthermore, the evaluation of the maceration of dried leaves of *Pinus caribaea* showed that the larvicidal activity was correlated with the concentration of lignin (Kanis et al., 2009).

2.5. Green plastics

Global demand for renewable and biodegradable plastics (green plastics) has increased due to a decrease in the supply of fossil fuels and concern for environmental issues. Naturally available terpenoids, such as β-pinene, have been reported as precursors for substituted ϵ-caprolactones (Quilter et al., 2017). These results are relevant from a sustainability point of view, since caprolactone is generally made from crude oil and serves as a key precursor for the synthesis of green plastic polymers (Schmidt et al., 2015).

According to Zhu et al. (2016), the use of renewable resources in the production of polymers is increasing, especially terpene monomers, which can be used as raw materials for the manufacture of a variety of materials and products. An example is turpentine, which contains α-pinene, β-pinene and minor amounts of other monoterpenes such as limonene (Gandini and Lacerda, 2015). Currently, the thermoplastic elastomers are derived primarily from petrochemicals used for various applications such as car suspension systems, window seals, household or electronic product coatings, shoe soles or medical devices and asphalt (Bolton et al., 2014; Zhu et al., 2016). Bolton et al. (2014) have shown that two pinene-derived monomers, α-methyl-p-methylstyrene and myrcene, can be incorporated into ABA tri-block copolymers (PAMMS-*b*-PMYR-*b*-PAMMS). These novel copolymers showed acceptable mechanical strength and elasticity compared with thermoplastic elastomers.

Another boost for the sustainable growth of the polymer industry is the bio-production of styrene. Styrene is one of the main building blocks of various polymeric materials commercially available and produced mainly from petroleum (Azeem et al., 2013; Sarkar and Bhowmick, 2016). Azeem et al. (2013) studied the production of styrene from *Penicillium expansum* cultivated on forest waste biomass such as leaves, wood, soft bark and mature bark of *Pinus sylvestris*, *Quercus robur*, *Picea abies*, and *Betula pendula* in the presence of yeast extract broth. The results suggested that the fungal strain could be used to produce “green” styrene plastics using renewable forest waste biomass, including that from pines.

The use of natural fibers as reinforcement in composite materials has been of increasing interest in industrial applications (Arrakhiz et al., 2013b). The benefits of using natural fibers in polymeric materials are the biodegradability, abundance and cost-effectiveness (Arrakhiz et al., 2012). Despite of the fact that pine cone fibers were rarely used as reinforcement in polymer composites, studies have shown that the fibril morphology and the good mechanical properties of this plant organ allow its use as filler material (Arrakhiz et al., 2012). Costa et al. (2017) evaluated the potential of pine cone fibers (*Pinus elliottii*) as filler in composites using polyurethane as matrix composites and also an alternative alkaline surface treatment to improve interfacial fiber/matrix adhesion. The results showed that the flexural strength and modulus of the composites were influenced by the fiber concentration, pretreatment and the size of the fiber particles. In the study by Arrakhiz et al. (2012), pine cone (*Pinus pinea* L.) fibers were used as new matrix reinforcement in a polypropylene matrix. Fiber–matrix adhesion was assured by the use of triblock copolymer of styrene-(ethylene-butene)-styrene grafted with maleic anhydride. Arrakhiz et al. (2013a) also elaborated polypropylene/pine cone fiber/clay composites and found that the addition of clay to polypropylene/pine composites improved tensile properties.

A growing concern today is the disposal of organic waste. According to Das et al. (2015), as regional councils and the waste management sectors strive to prevent, mitigate and reverse adverse effects of land-based organic/wood waste, more attention has been paid to the development of alternative solutions. Organic waste (*e.g.* agricultural and forestry wastes) can be converted into a carbonaceous material (biochar), produced when organic wastes are heated at high temperatures (~500 °C) in oxygen limited conditions (pyrolysis process) (Das and Sarmah, 2015; Das et al., 2016; Kookana et al., 2011). Biochar is a renewable, sustainable, cheap, non–toxic material that currently has no value-added applications beyond conventional carbon sequestration, soil alteration, and contamination removal (Das et al., 2015). On the other hand, studies have shown uses for biochar in polymer composites. Das et al. (2015) attempted to find out the most suitable loading amount of *Pinus radiata* wood derived biochar in wood/polypropylene composites. The authors observed that a loading amount of 24 weight percent was the most appropriate for enhancing the mechanical properties of the composites.

2.6. Nanotechnology

Nanofibrillated celluloses (NFC) have drawn much attention because of their unique physicochemical properties and wide number of applications (Hu et al., 2017). Nanofibrillated cellulose is described as a long and flexible cellulosic material with a diameter lower than 100 nm, obtained from cellulose fiber by various methods (Missoum et al., 2013). As an example, Xiao et al. (2015) used natural pine needles to extract cellulose, which was then used to isolate aqueous NFC suspensions by combining acid-pretreatment with high-intensity ultrasonic treatment. The diameters of the produced pine needle nanofibers were within the range of 30-70 nm and exhibited improved thermal properties, making them promising candidates for use in thermoplastic composites.

Despite of the fact that silica nanoparticles (NPs) have drawn widespread attention due to their applications in many emerging areas, the conventional methods for synthesizing silica NPs are expensive and use non-renewable precursors. In this sense, Si-accumulation plants may be a renewable alternative – cheap and eco-friendly – to produce biogenic silica NPs (Mattos et al., 2016). One of these alternatives was described by Assefi et al. (2015) in which nanosilica particles were successfully synthesized by thermal decomposition of pine cones and pine needles. The silica NPs were obtained after sulfuric acid treatment and calcination temperature of 600 °C for 3 h (Assefi et al., 2015).

Silver nanoparticles (NPs) have increasingly been used in various fields as dressings for wounds, coatings for medical devices and impregnated into fabrics, besides other pharmaceutical and food industries applications (Rai et al., 2009). Velmurugan et al. (2015) established an efficient method for the biosynthesis of AgNPs from an aqueous silver nitrate solution using young cone extract of *Pinus densiflora*. According to Velmurugan et al. (2015) various phytochemicals present in the leaf extract could play a key role in the conversion of the ionic form of silver to the metallic nano-form. In addition, the synthesized AgNPs proved to be moderately effective against four kinds of gram-positive skin bacteria.

3. Special materials

3.1. Fiberboards and water treatment devices

Pine cones are one of the most common residues of lignocellulosic forest in North America, and large quantities are produced worldwide, especially in pine plantations grown for pulp and paper industries (Ayrilmis et al., 2010). They are mainly composed by cellulose, lignin and resins, which contain a variety of organic compounds (Altundoğan et al., 2016; Ayrilmis et al., 2009). After collecting seeds, cones are usually discarded or burned for energy (Rambabu et al., 2016). Various studies reported the effective use of *Pinus pinea* cones in the fabrication of medium density fiberboard (MDF) and the significant reduction of formaldehyde emission during production using a combination of wood fiber / cone flour in various proportions (Ayrilmis et al., 2009). Studies on the manufacture of wood-plastic composites (WPC) showed that addition of 10 % (w/w) of cone flour did not affect significantly WPCs flexural properties and water resistance, being comparable to those produced with wood flour ((Ayrilmis et al., 2010). In another study, particles from stone pine cones were shown to be an alternative to wood material in the manufacture of particleboard used in indoor environments due to lower thickness swelling, water absorption and lowering formaldehyde emission (Buyuksari et al., 2010).

A study by Altundoğan et al. (2016) demonstrated that pine cones are more suitable than cellulose-based agricultural byproducts to obtain an ion exchange material, due to some physicochemical properties of the material such as water retention, swelling capacities and mechanical resistance. An ion exchanger from pine cone was developed by citric acid modification and its water hardness removal properties were evaluated. The modified cation exchanger obtained from pine cone proved effective for the removal of hardness from water as a cheap, durable and environmentally friendly material.

Currently, pine cone biomass has also been applied as a biosorbent to waste water contaminated with metals and paints (Aksakal and Ucun, 2010; Ofomaja and Naidoo, 2011). Ofomaja and Naidoo (2011) studied the sequestration of copper from an aqueous solution using pine cone biomass, showing it was very efficient, removing a high percentage of pollutant in a short period of contact. The biosorption capacity of pine cone was improved with $Ca(OH)_2$, KOH and NaOH, obtaining the best results with the latter (Ofomaja and

Naidoo, 2011). Pine cone shell can also be used as biosorbent of nickel and other heavy metals (Almendros et al., 2015; Blázquez et al., 2012; Martín-Lara et al., 2016; Ofomaja and Naidoo, 2011; Ofomaja et al., 2010). Ofomaja and Naidoo (2011) pointed out that a great advantage of the use of pine cone powder as a biosorbent is the fact that a large amount of metal ion from the solution can be removed in a relatively short time. Aksakal and Ucun (2010) showed that cone biomass of *Pinus sylvestris* has potential use as an alternative biosorbent material for the removal of reactive red dye from aqueous solutions.

Pine cone is a lignocellulosic forest residue with aggregate industrial importance in terms of energy and material production. Supercritical water, *i.e.* water above critical temperature ($T_C \geq 374$ °C) and critical pressure ($P_C \geq 22.1$ MPa), has high kinetic energy and densities similar to gases and liquids, respectively. When used in gasification, supercritical water has the ability to completely dissolve organics and gases (Nanda et al., 2017). Nanda et al. (2017) investigated thermal events leading to the decomposition of pine cone in subcritical (300 and 350 °C, 21 MPa), almost critical (370 °C, 22 MPa) and supercritical (450 and 550 °C, 23 MPa) conditions. They identified pine cone as a promising raw material for supercritical water gasification (high H_2 and total gas yields), suggesting that gasification in supercritical water can lead to the generation of syngas rich with H_2, hydrochar with high carbon content for environmental applications, and liquid effluents enriched with industrially relevant oxygenated products.

3.2. Pine bark

Pinus bark has also been used as an alternative for the removal of metals in contaminated water. According to Daniel Schwantes et al. (2018), the contamination of water by toxic metals is one of the most important sources of pollution, as these metals have a high degree of toxicity to humans and the environment, even at low concentrations. These authors aimed at adding value to *Pinus* bark by chemically modifying its raw material (sieved particles) to acquire adsorbent characteristics favorable for its use to adsorb Cd, Pb and Cr. Results showed that adsorbents based on modified *Pinus* bark are excellent alternatives for removal of these metals from contaminated water.

3.3. Pine stalks

The use of dyes by the textile industries generates a large amount of toxic wastewater that needs to be treated before entering into the aquatic environment (Hu and Jefferson, 2002; Jafari et al., 2017). Jafari et al. (2017) analyzed different sizes of activated carbon from *Pinus eldarica* stalks as a support for deposition of ZnO NPs. The resulting low cost adsorbent can be used for treatment of wastewater containing azure II and of auramine O dyes.

3.4. Membrane permeation

Addition of skin penetration enhancers in a formulation is the simplest and most common technique to improve transdermal permeation of drugs (Behtash Oskuie et al., 2018). Behtash Oskuie et al. (2018) developed novel vesicular systems of liposomes and ethosomes using turpentine as a penetration enhancer to improve fluconazole skin permeability. Results showed that these novel formulations have the potential to improve transdermal delivery and antifungal activity and they may be considered promising carriers to improve fluconazole permeability. In addition, turpentine itself may contribute for fungal control.

5. Conclusions

Non-wood pine tree biomass has multiple and ever increasing applications in different industrial sectors such as chemicals, pharmaceuticals, food additives, agrochemicals, landscaping, bioenergy, construction materials, water waste treatment, nanotechnology products, green plastics and batteries. Essentially every component of pine trees can contribute for generating one of more products from this array of uses (Fig. 4).

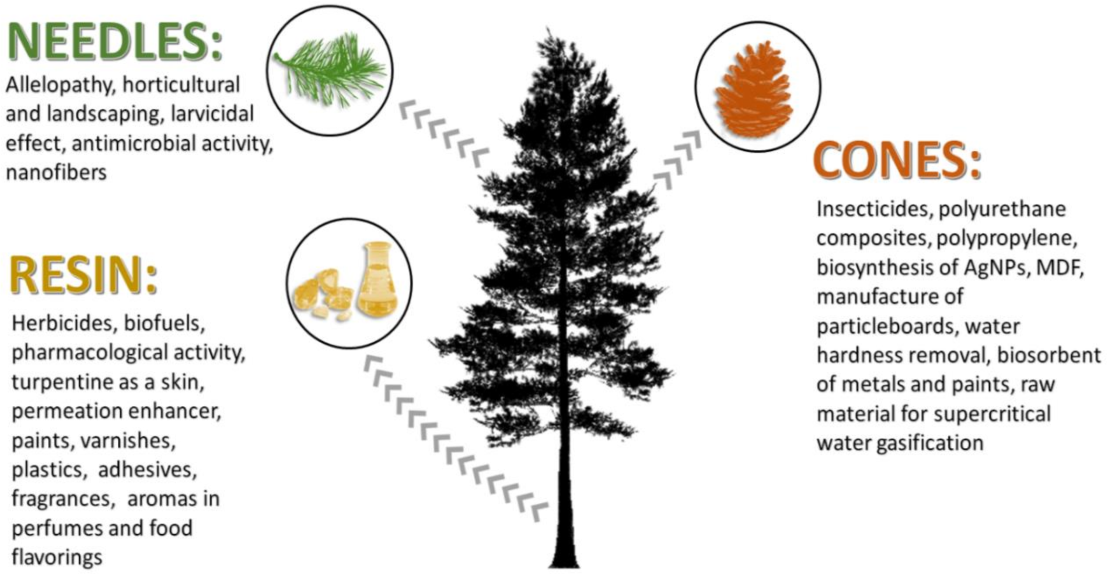

Fig. 4. Multiple uses of needles, cones and pine resin.

A prominent role is played by pine resin and its myriad derivatives, but other non-wood parts are increasingly important such as bark, needles, straw, and cones. Maximizing the exploitation of non-wood pine products can have a notable impact in promoting cleaner production alternatives to several products currently derived from fossil fuels, as well as help stabilizing forest property incomes and making this activity even more sustainable. As continued research efforts to develop pine derived products unfold, boundless possibilities for this major tree crop can be expected.

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CAPÍTULO 2

Resin exudation profile, chemical composition and secretory canal anatomy characterization in contrasting yield phenotypes of *Pinus elliottii* Engelm.

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Resin exudation profile, chemical composition and secretory canal anatomy characterization in contrasting yield phenotypes of *Pinus elliottii* Engelm.

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ABSTRACT

In conifer stems, secretory canals synthesize and store resin, which acts as a major defense mechanism against herbivores and pathogens. Resin terpenes are used as raw material by a large array of industrial sectors. Most forest stands operationally used in resin extraction are derived from seeds, displaying high variation in yield. The objective of this study was to identify adult *Pinus elliottii* genotypes of high yield of resin in a short timeframe by kinetic-volumetric flow analysis, aiming at the establishment of elite forests for resin tapping prior to its start. In addition, the anatomical basis of resin yield was investigated by examining the correlation between parameters such as number, shape, area and internal volume of wood canals with resin production. Monoterpene composition in resin of high and low yielding trees was also compared. The resin flow-based selection method was reliable for resin yield phenotype detection, confirming this property in trees formerly identified as being of high and low resin production by conventional tapping. The reverse test for identification of high and low yield resin features in previously untapped younger plants was in good agreement with their yields after subsequent standard tapping procedure. To evaluate and quantify the three-dimensional structure of resin canals, we used microCT scans. The number of axial resin canals was significantly higher in the high resin yielding trees when compared to low yielding ones. Frequency of anastomosed canals and canal diameter were also superior in the former. Chemical analyses of resin monoterpenes revealed that the ratio of α-pinene/ β-pinene was higher in more productive trees, which also had more limonene in total terpenes compared to the low yield counterparts. Data support the use of short-term kinetic-volumetric analyses as a tool to identify and select high yield trees for the establishment of elite slash pine forests for resin tapping operations and show that the superesinous phenotype strongly correlates with canal density and structure.

Key words: *Pinus*, resin canals, tapping, resin composition, high yield, microCT.

1. Introduction

Conifers have developed a series of adaptive strategies to deal with herbivore and pathogen attacks (Franceschi et al., 2005; Keeling and Bohlmann, 2006; Geisler and Jensen, 2016). Resin is considered the major defense of conifers, and its composition consists of various terpenoids such as monoterpenes, sesquiterpenes, and diterpenes (Phillips and Croteau, 1999; Martin et al., 2002; Zulak and Bohlmann, 2010).

Resin is synthesized and accumulated in specialized secretory structures (isolated resin cells, multicellular resin blisters and networked resin ducts), which may appear as a normal feature of development in tissues (constitutive defense) or may result by the induction of external factors (Bannan, 1936; Lewinsohn et al., 1994; Wu and Hu, 1997; Hudgins et al., 2003; Langenheim, 2003). A related, commonly induced response to mechanical damage, insect attack, fungal invasion, application of hormones and chemical stimulants is the production of traumatic resin ducts in the xylem (Lombardero et al., 2000; Nagy et al., 2000; Franceschi et al., 2002; Arbellay et al., 2014). The formation of traumatic resin ducts represents an important induced defense that enhances resin production and flow in response to environmental perturbations in tissues close to the wounded zone (Franceschi et al., 2005; DeRose et al., 2017).

Resin canals of Pinaceae are differentiated into radial canals and axial canals, depending on their orientation in plant tissues, creating a complex network (Bannan, 1936; Lewinsohn et al., 1991; Rodríguez-García et al., 2014). Resin flow can be influenced by an array of factors such as irradiance, temperature, season, and edaphic conditions, as well as by genetics, age, and wounding (Peñuelas and

Llusià, 1999; Ayres and Lombardero, 2000; Knebel et al., 2008; Rodrigues and Fett-Neto, 2009; Hood and Sala, 2015; Neis et al., 2018). In addition, anatomical variation in xylem tissues and cells such as resin canals may alter resin content (Keeling and Bohlmann, 2006; Ferrenberg et al., 2014). As with resin flow, resin duct development is also influenced by genetics, climate and environmental factors (eg. soil fertility and disturbance) (Rosner and Hannrup, 2004; Hood et al., 2015; Moreira et al., 2015) and after mechanical (Rodríguez-García et al., 2014), chemical (Moreira et al., 2015) or fire-induced wounding (Hood et al., 2015).

In Southern Brazil, *Pinus elliottii* Engelm var. *Elliottii* (slash pine) is extensively cultivated, accounting for approximately 24% of the annual resin production (ARESB, 2016). Tapping pine resin involves periodic wounding of the trunk and stimulation of both resin production and flow. Physiological and molecular aspects of this process have been investigated aiming at increasing yields (Rodrigues et al., 2011; De Lima et al., 2016). The genetic improvement of forests is especially geared towards higher production of quality resin, but most forests have been grown essentially for timber. Paradoxically, the resin sector has often become economically more attractive, in part as a function of expanding uses of resin-derived products (Rodrigues-Corrêa et al., 2012). The average market price of Brazilian resin in recent years is circa US$ 1,100.00 per ton (ARESB, 2016), with each tree yielding, on average, 2.5 to 3.0 kg of resin per year.

Research to optimize the production of induced resin by stimulating pastes has been carried out, yielding improvements with the use of new adjuvants of lower cost compared to the standard commercial paste, essentially composed of sulfuric acid and 2-chloroethylphosphonic acid, a synthetic ethylene precursor (Fett-Neto and Rodrigues-Corrêa, 2012).

The significant market value and global demand of resin and its main fractions (rosin and turpentine) and the increasing socioeconomic and environmental importance of the tapping activity motivated the present investigation. The overall goal was to provide tools for selection of elite genotypes from current slash pine stands for resin tapping and, at the same time, increase the understanding of resinosis in a commercially relevant pine species for the resin industry. The specific objectives of this work were: a) establish and validate a fast identification method of high resin yield individuals using kinetic-volumetric analysis; b) determine resin composition associated with high production phenotype, c) and investigate the correlation between number, area and internal volume of secretory canals with resin production.

2. Materials and methods

2.1. Plant material and study area

Slash pine (*Pinus elliottii* Engelm. var. *elliottii*) trees with approximately 18-years old and a diameter at breast height ranging from 65 to 90 cm, grown in pine forests in Rio Grande do Sul, city of Balneário Pinhal (approximately 30.17° S, 50.20° W) were used in the experiments. The trees selected for this study had been tapped for two years with application of paste containing CEPA (2-chloroethylphosphonic acid) prior to the experiments herein described and their yields were individually recorded over that period. The forests were located in the installations of the forest company Celulose Irani S.A.

2.2. Resin sampling

Twenty trees of *P. elliottii* were selected based on stimulant paste induced resin production and conventional tapping. Half of them were considered high yield trees (producing more than 8.70 kg per tree annual) and the other half low resin yield trees (production of less than 3.93 kg per tree annual) (Supporting Information, Fig. S1). On each selected tree, three alternating holes were made with an increment borer in the opposite face of the commercial streak panel, with approximately 4 cm of depth and 45° upward angle, to facilitate the resin flow. Each hole was drilled at the same height (breast height), keeping a distance of 60 degrees between the holes. A cylindrical tube of 12 mm diameter attached to a plastic bag was inserted in the hole for resin collection (Supporting Information, Fig. S2). The tubes were kept in the trees for four hours. After this period, the tubes were collected and exuded resin was weighted. Experiments were performed twice independently, in February 2016, always in the afternoon.

As additional validation, the same experiment was performed with young trees (approximately 9-years-old), never previously tapped, in May 2016, also in the afternoon. A total of 100 trees were evaluated for 4 hours for the identification of high and low yielding trees, based on the amount of exuded resin in collector tubes. The methodology was the same as described above. According to the results obtained (Supporting Information, Fig. S3), 15 trees of each phenotype (high and low yield) were chosen for seasonal evaluation.

In this experiment, bark strips, 2.5 cm wide and 2–5 mm high, were removed every 15 days, with application of a paste containing CEPA (2-chloroethylphosphonic acid) in approximately one-third of the tree circumference, exposing the sapwood surface. The seasonal resin production was evaluated between the years of 2016 and 2017. Plastic bags were placed at the base of the wounds to collect the exuded resin; bags were regularly checked and replaced when needed to avoid resin loss. The bags containing resin were weighed on a field digital balance after careful removal of rainwater (Rodrigues et al., 2008).

2.3. Wood core sampling

Wood cores were randomly sampled from high- and low- yielding trees as previously identified by resin sampling (section 2.2). Six trees were selected among those characterized as high yielding resin producers and another group of 6, among the low resin producing individuals. Three wood core samples (5 mm diameter x 18 mm length) were extracted with an increment borer from each tree at breast height and at 45° upward around the stem, exactly as resin sampling was done (Supplementary video 1). Extracted wood cores were soaked in 100% acetone according to Westbrook et al. (2015) and freeze-dried for 24 h to extract any resin residue before imaging. The use of this technique did not allow the analysis of the radial resin canals, only the analysis of the axial resin canals.

2.4. X-ray Microtomography (μCT)

Resin canal traits were measured from cross-sectional X-ray images (3D) of wood cores that were obtained by using a high-resolution X-ray microtomograph (SkyScan 1272 Bruker microCT, Kontich, Belgium). X-ray source voltage and current were 20 kV and 175 μA, using an objective lens of 4×, resulting in a voxel size of 10.000188 μm. Stack image processing was analyzed using a Sigma plugin filter in the open source FIJI/ImageJ software (Supplementary video 2). Each resin canal was manually segmented using the Multiple Slice Edit tool of Mimics software (Materialise®, Leuven, Belgium). The process consists of drawing a mask over a specific tissue in a number of parallel sections along the stack and later interpolating the mask in all other sections. The greater the number of manually masked slices before interpolation the higher is the similarity with the sample's actual 3D geometry (Palombini et al., 2016). This method was used to generate masks into the whole stack, segmenting the sample into the resin canal regions. Segmented regions allowed further numerical analysis. As a result of the freeze-drying process, resin canals quantified and measured in these experiments did not contained epithelial and parenchymal (subsidiary). Axial resin canals were visually counted in early and latewood images to obtain resin canal number. Frequency of axial resin canals (ARC) (number of canals per mm2 of cross section) was measured according to the area of the growth rings. For analysis of volume, mean cross-sectional area and diameter of resin canal, the wood cores were divided into three parts (Supporting Information, Fig. S4). In each part, three canals were selected for detailed assessment with BoneJ's Particle Analyzer plugin (Doube et al., 2010) or FIJI/ImageJ.

2.5. Resin analysis

The same trees used for resin sampling and wood-core sampling were also used for a qualitative and quantitative resin analysis. Samples were collected in the Fall of 2014 directly into microcentrifuge tubes, which were immediately frozen in liquid nitrogen and kept as such until storage at −80 °C. Preparation of samples for terpene analysis was performed as previously described (Wang et al., 1997). Six biological replicates of each phenotype were evaluated and extractions and analyses were done in triplicate. Gas chromatography (Gao et al.) and GC-Mass Spectrometry were used for quantitative and qualitative analyses of resin, respectively, as previously described (Rodrigues et al., 2011).

2.6. Soil data

The soil in the area is classified as Hydromorphyc Planosoil, Eutrophic Solodic (Streck et al., 2008). No fertilization was applied throughout the study.

2.7. Statistical analyses

Results were analyzed by ANOVA followed by Tukey test and Student’s t-test, whenever appropriate, using the statistics package SPSS 20.0 for Windows (SPSS Inc., USA). Data were expressed as mean ± standard error (S.E.).

3. Results

The kinetic-volumetric analysis was performed after the drilling damage in previously selected trees according to their level of resin production during tapping in previous years (Supporting Information, Fig. S1). Results confirmed that trees of high yield produced significantly more resin in a period of 4 hours than the trees of low yield. Considering the average of two evaluations, high yield trees exuded 16.53 g of resin whereas those of low yield produced 6.29 g (Fig.1).

Next, the same technique was evaluated in younger plants (approximately nine years old), which had never been tapped before in order to evaluate the effectiveness in identifying trees of high and low resin yield. Subsequent tapping experiments in those trees have confirmed the phenotypes obtained with kinetic-volumetric analysis and maintained the same responses throughout seasonal evaluations performed during one year (Fig. 2). The analysis of seasonal variation of exuded resin showed that highest yields were observed in Summer and Spring (1.61 and 1.60 kg, respectively) (Fig. 2). The seasonal results corroborated the profile already seen in 18-year-old *P. elliotti* trees (Neis et al., 2018).

These findings indicate that the kinetic-volumetric method is reliable for the identification of genotypes with resin yields higher than the average of a tree population, with the major advantage of selecting trees in a shorter period of data collection, lower cost and being suitable for the evaluation of young plants, which have not been used in commercial tapping.

Analyses to better understand the structural bases involved in resin production and exudation processes in both high and low yield trees were also carried out. Compared to the equivalent low resin yield individuals, eighteen-year-old trees with high resin yield showed higher axial resin canals frequency (0.298 versus 0.130/mm^2), which also had larger area (0.044 versus 0.024 mm^2), diameter (279.282 versus 157.651 µm) and volume (0.011 versus 0.003 mm^3) (Fig. 3).

For a more detailed analysis, wood cores were separated into three parts (outer; mid; inner) and the area, volume and diameter of the resin canals were evaluated. The three parts of cores from the low yield trees were not statistically different for any of the parameters quantified (Fig.4). On the other hand, the cores of the high resin yield trees showed statistical difference between the parts only in relation to diameter. The outer part of the core presented resin canals with a larger diameter when compared to the inner part (Fig.4A).

The relationship between phenotype (low- and high-resin yield) and the quantified parameters in the different parts of the wood was consistent. High yield trees showed higher values for all anatomical variables when compared to the low yield trees (Fig.4). These results were corroborated by correlation analysis, which revealed that resin production was positively correlated with diameter ($r = 0.637$; $p < 0.000$), frequency ($r = 0.534$; $p < 0.000$), volume ($r = 0.444$; $p < 0.000$), and area ($r = 0.393$; $p < 0.01$) of resin canals (Fig.5). Areas closest to cambium showed traumatic resin canals, probably as a result of the wounding (Supporting Information, Fig. S5). High resin yield trees also presented anastomosed axial resin canals (Supporting Information, Fig. S6).

The analysis of monoterpenes in resin turpentine of high yield trees showed that the main components were β-pinene and α-pinene, respectively (overall average in % of total turpentine terpenes β-pinene = 43.6; α-pinene = 39.6). An opposite profile was found for the low yield trees (overall average in % of total turpentine terpenes: α-pinene = 49.5; β-pinene = 35.9). The proportion of α and β-pinene in turpentine was significantly different depending on resin yield of the trees (Fig. 6). Higher α/β-pinene ratio was found in the low yield trees when compared to the high yield ones (Fig. S7). Camphene and limonene were the other components detected in the samples (Fig. 6). The amount of limonene was higher in the high resin yield trees. The proportion of camphene was very similar in high and low resin yield trees, ranging from 0.48% in high yield trees to 0.58% in the low yield trees (Fig. 6).

4. Discussion

In the resin extraction process, many of the inducible responses are results of alterations in gene expression that influence the biochemical regulation of synthesis, catabolism, conversion and transport of secondary metabolites. Inducible responses occur as a function of the environmental stimulus (injury and chemical stimulation) and plant genotype (Lombardero et al., 2000). Structural aspects of trees such as the tree diameter, percentage of live crown, number and size of resin ducts in the xylem and phloem also play key roles in the production, storage and transport of resin (Hood and Sala, 2015).

Studies on gene expression changes during resin production have shown that in *Pinus massoniana* (27 years-old) not treated with stimulant paste, a high level of expression of a monoterpene synthase was associated with the increase of resin production (Liu et al., 2015). In *P. elliotti*, increased resin yields in treatments stimulated with phytohormone-based pastes (auxin NAA and ethylene precursor CEPA) were consistent with higher expression of terpene synthases (α-pinene synthase, β-pinene synthase and abietadiene synthase) (De Lima et al., 2016). Resin-related transcript changes often correlate with resin production.

Currently, most of the existing methods to determine resin production profile in adult slash pine trees require tapping operations (Rodrigues et al., 2008; Rodrigues and Fett-Neto, 2009; Rodrigues et al., 2011; Rodrigues-Corrêa and Fett-Neto, 2013) or laboratory chemical analyses (Karanikas et al., 2010). These are laborious and time-consuming practices and/or may require expensive specific equipment. The most common method for the identification of genotypes with high resin production is the removal of 2.5 - 5.0 cm of bark stripes (twice a month) with or without the use of chemical stimulants. The resin extracted from the resin canals is collected in several types of containers (plastic bags, metal containers) that are attached with a wire or rope around the base of the wound. To stimulate and maintain resin flow, a slurry containing sulfuric acid is applied to the fresh surface of the wound (Clements, 1970). A major drawback is that seasonal assessments take at least one year to have the accurate recognition and selection of individuals with high resin potential. For example, (Rodríguez-García et al., 2014) evaluated 4 years to be able to differentiate *Pinus pinaster* trees of high and low yield resin.

To the best of our knowledge, this is the first systematic and validated analysis for selection of higher producing adult trees on a commercial setup based on a kinetic-volumetric technique, which can have an important role in the management of forests established from seeds, as is the case of most slash pine stands. Natural tree regeneration from seed banks is considered a cost-effective way to re-establish vegetation and help preserve genetic identity and diversity (Yang et al., 2014). As plants develop, regenerated forests must be thinned. This process is very important for the proper management of softwood stands (Picchio et al., 2012). Considering that this removal normally does not take into account resin yield, the use of kinetic-volumetric method before thinning can improve this process, allowing to select trees with higher resin productivity in a significantly shorter period of time compared to conventional tapping (from years/months to hours/days). In addition, details on *P. elliottii* defense system, specifically on the structures and processes that can affect resin production, can also be useful for selecting trees with superior quality for this economic activity.

Anatomical variables herein examined (axial resin canal frequency, diameter, area per mm^2 and the total volume of resin canals per growth ring) appear to influence resin production in slash pine. Similar findings were also described for Norway spruce (*Picea abies*), in which resin flow was highly correlated with both the total number of resin canals as well as the total duct area (Netherer et al., 2015). (Luchi et al., 2005) observed an increase in channel formation correlated with the increase in resin production in response to a wound induction and the inoculation of different fungi in *P. nigra* (Liu et al., 2013; Rodríguez-García et al., 2014) reported that resin yield is strongly correlated with tree diameter, crown percentage, radial resin channel number and resin channel volume. Indeed, it has been noted that number and size of resin ducts in the xylem and phloem are important in resinosis, as they are responsible for the production, storage, and transport of terpenes (Hood and Sala, 2015).

Several examples of positive correlation have been found between resin flow and resin canals (Bannan, 1936; Rosner and Hannrup, 2004; Ferrenberg et al., 2014; Westbrook et al., 2015). Conifers exhibit resistance to herbivores and other wounds through carbon-based compounds that are synthesized and accumulated in tissues at high concentrations (Franceschi et al., 2005). These defenses require large amounts of carbon resources, resulting in potential competition with other plant functions (Sampedro 2014; Villari et al., 2014). Perhaps low-yield resin trees have invested more in growth rather than canal differentiation and resin biosynthesis, thereby affecting the defense mechanism. Future studies should address this possibility.

The relationship between the yield of resin and the concentration of each monoterpenoid in turpentine obtained from resin was also investigated. Data indicated that some compounds can be used as diagnostic marker for high resin yield trees, as described for *P. halepensis* (Karanikas et al., 2010). However, in our study it was not possible to identify a single monoterpene as a marker. On the other hand, it was observed that high yield trees presented higher amount of β-pinene when compared to the low yield ones. The former trees also had higher content of limonene. This chemical profile of high yield trees has relevant implications since β-pinene is the isomer that has higher value for the chemical industry and limonene also finds several economic uses (Rodrigues-Corrêa et al., 2013). Higher expression of β-pinene synthase was observed in paste stimulated tapped slash pine trees (De Lima et al., 2016).

Resin terpene synthesis in conifers is known to be under genetic control with variation occurring within and between populations (Trapp and Croteau, 2001). According to Sampedro et al. (2010) chemical defenses can be modulated by the environment and by the interactive response of genotypes to environmental conditions such as resource availability. The data herein described support a relatively fast means to capture elite genetic material from heterogeneous stands for the specific purpose of resin extraction, allowing the establishment of more homogeneous and productive forests.

5. Conclusions

The kinetic-volumetric method is suitable for the early identification of high resin yield trees and subsequent selection and/or clonal multiplication of superior genotypes for the implantation of commercial forests with the main purpose of resin extraction. Higher yield of resin is positively correlated with number, diameter, area and volume of the resin canals, highlighting the importance of structural basis for resinosis. Turpentine composition between high and low resin yield trees is different, the former being characterized by a higher presence of β-pinene and limonene.

This information may be helpful for tapping management and establishment of resinosis-directed forests, since stands made of high resin yield trees can afford increased productivity per area, maximizing resin biomass output and quality. In addition, the high resin yield parameters herein described can be used in resin-directed pine breeding programs.

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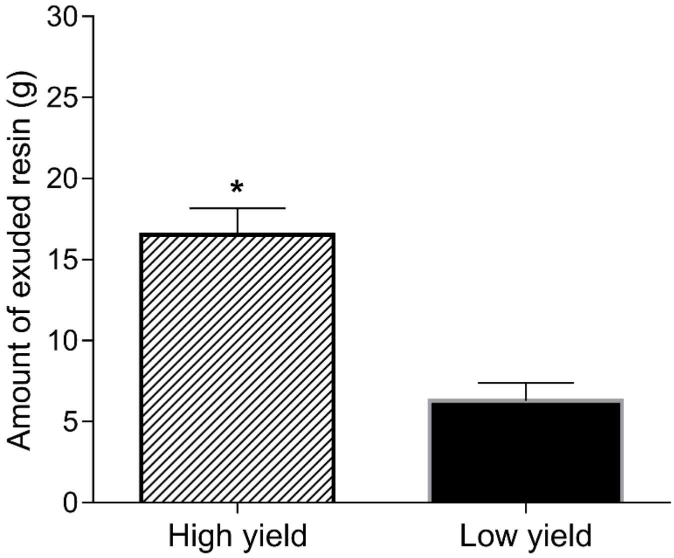

Fig. 1. Mean quantification of resin exuded after 4 hours in high and low resin yield trees (approximately 18-years-old) identified by conventional tapping. Standard errors are indicated on top of bars. Asterisks indicate significant difference according to Student's t-test, with $P \leq 0.001$.

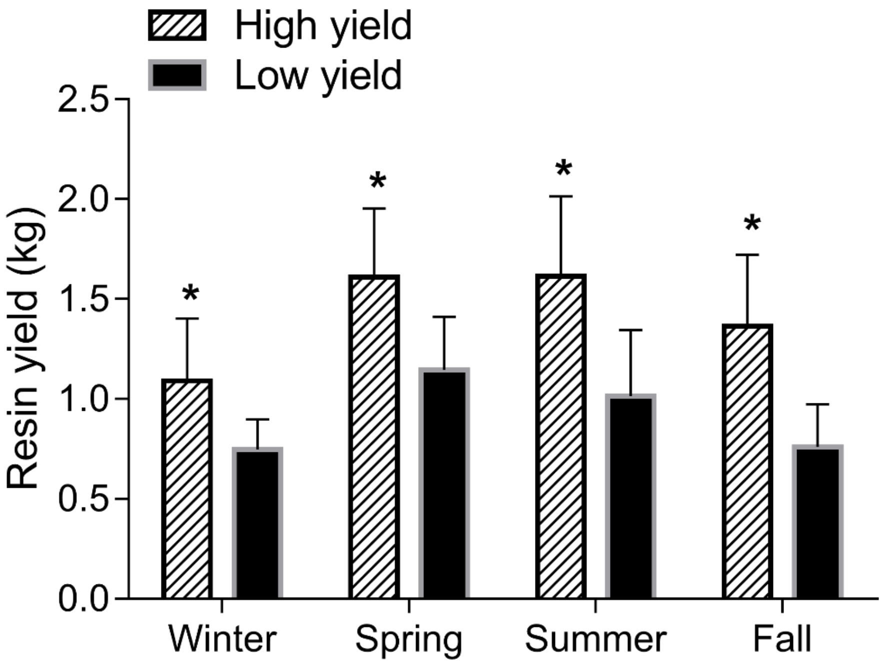

Fig. 2. Quantification of seasonal resin tapped from approximately 9-year-old trees identified as of high and low resin yield by kinetic-volumetric method. Standard errors are indicated at the top of the bars. The asterisks indicate significant difference according to the Student t test, with P ≤ 0.001.

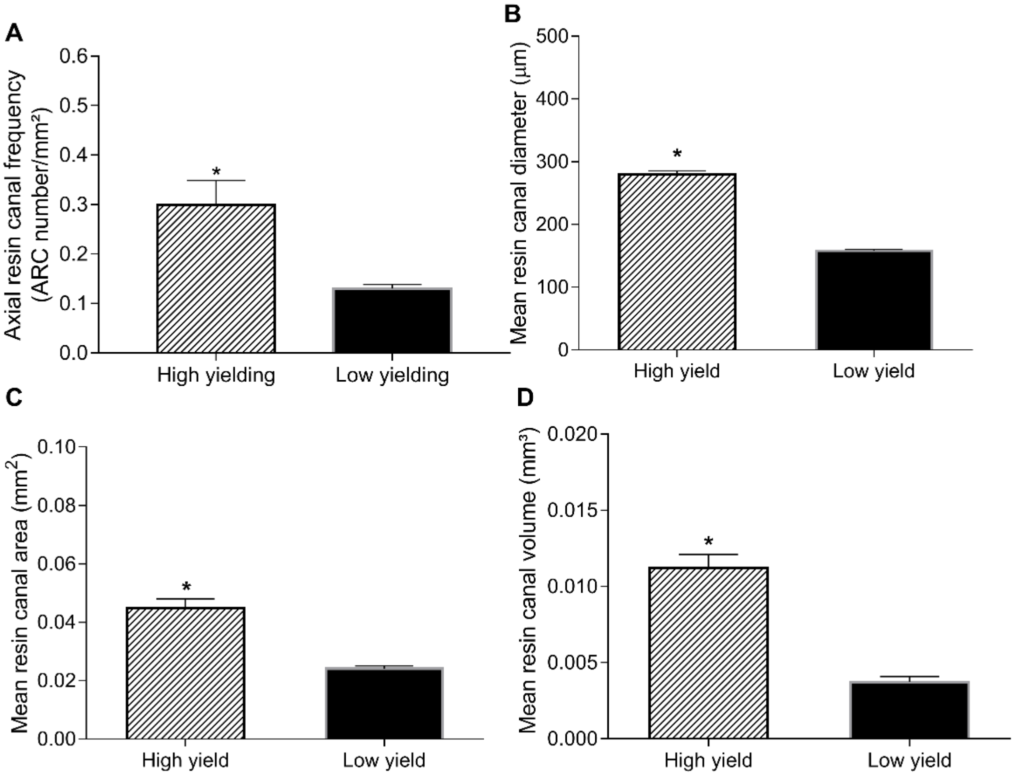

Fig. 3. Structural features of secretory canals in low and high-resin producing trees of *Pinus elliottii* (approximately 18-years-old). (A) Axial resin canal frequency (B) Mean resin canal diameter, (C) Mean resin canal area, and (D) Mean resin canal volume. Standard errors are indicated on top of bars. Asterisks indicate significant difference according to Student's t-test, with $P \leq 0.001$.

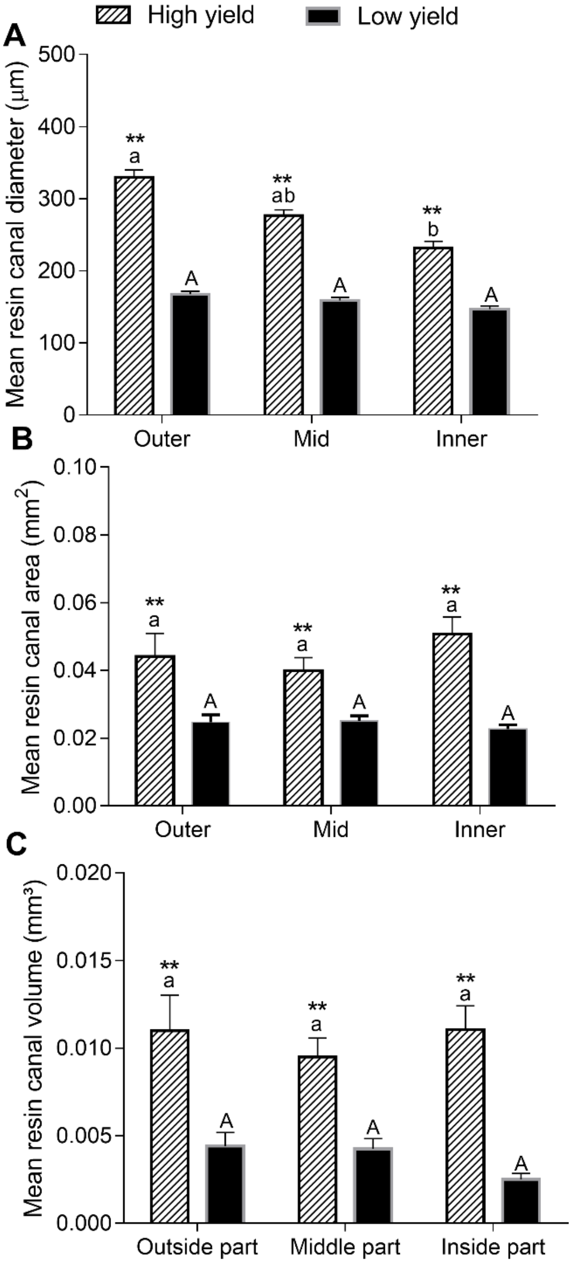

Fig. 4. Resin canal characteristics in outer, middle and inner portion of wood cores obtained from low and high-resin producing trees of *Pinus elliottii* (approximately 18-years-old). Standard errors of the means are indicated on top of bars. (A) Mean resin canal diameter (B) Mean resin canal area and (C) Mean resin canal volume. Different letters indicate significant difference among parts of wood cores

according to Tukey test ($P \leq 0.05$). Asterisks indicate significant differences between the phenotypes within each wood core portion, according to the Student t-test, with $^*P \leq 0.0001$.

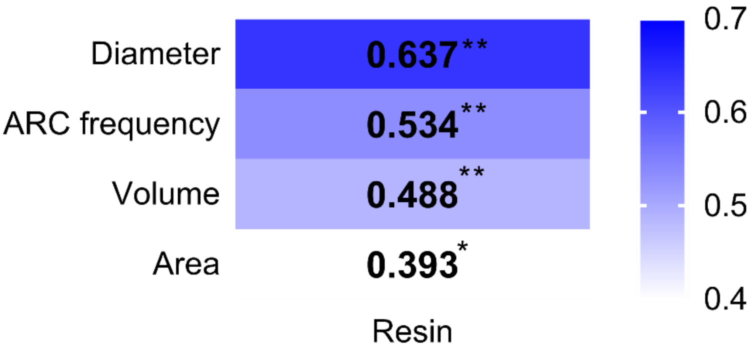

Fig. 5. Correlations between axial resin canal frequency, diameter, volume of canals, area and amount of exuded resin after 4 hours in trees of *Pinus elliottii* (approximately 18-years-old). Significant correlations are indicated by asterisks: *P < 0.01; **P < 0.0001.

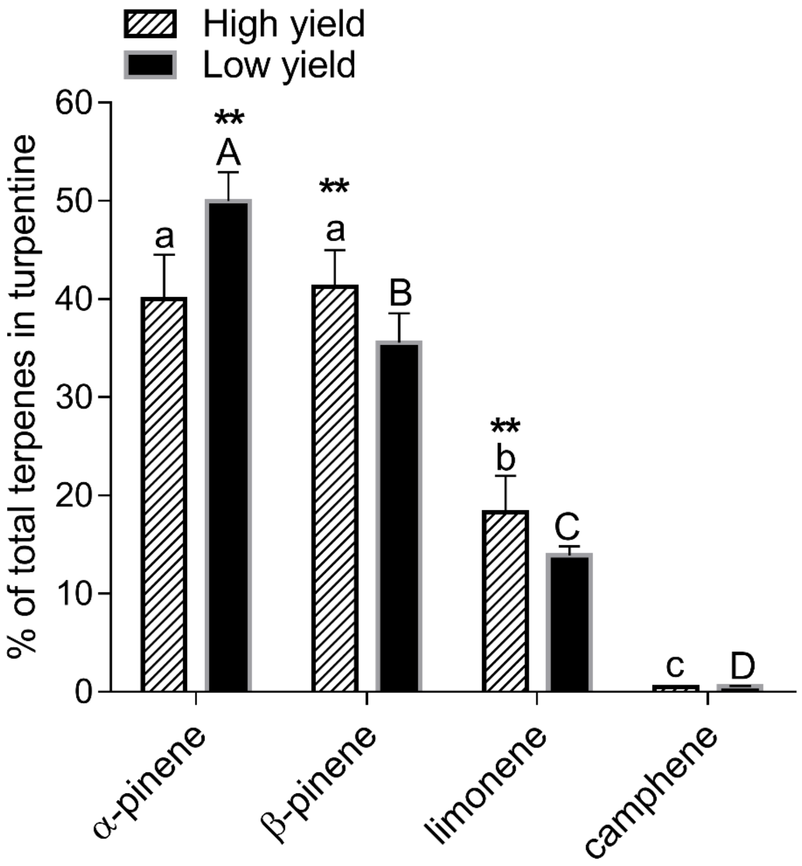

Fig. 6. Concentration (as % total terpenes) of monoterpenes in turpentine from high and low resin yield *Pinus elliottii* var. *elliottii* trees (approximately 18-years-old). Standard errors of the means are indicated on top of bars. Different letters indicate significant difference among monoterpene constituents within the same phenotype by Tukey test ($P \leq 0.05$). Asterisks indicate significant differences between the phenotypes within each monoterpene constituent analyzed, according to the Student's t-test, with $^{**}P \leq 0.001$.

Supplementary Information

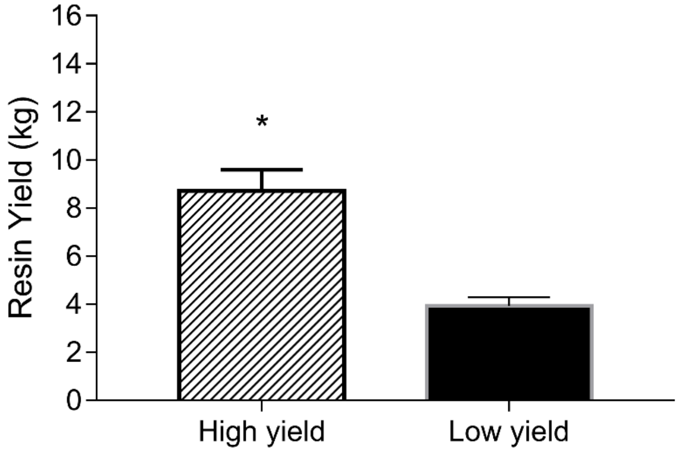

Fig. S1. Total annual production of resin in approximately 18 year-old-trees. Standard errors are indicated on top of bars. Asterisks indicate significant difference according to Student’s t-test, with P ≤ 0.0001.

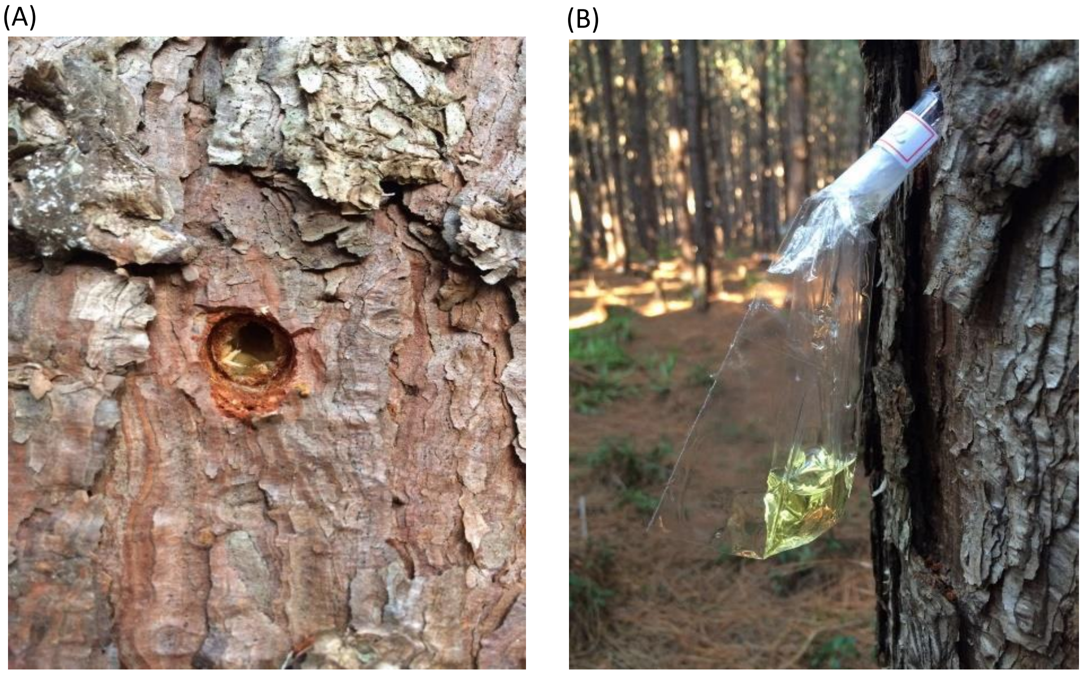

Fig. S2. *Pinus elliottii* trees (approximately 18 years old). (A) Detail of the hole made with the increment borer. (B) Tube coupled with a collecting bag, showing the resin exuded from the trunk.

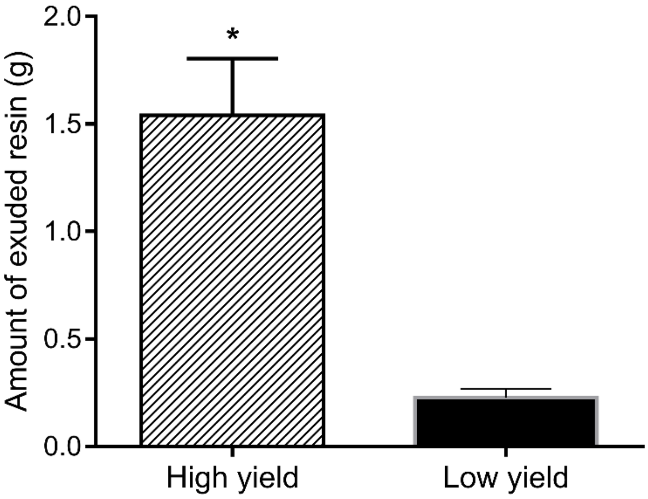

Fig. S3. Quantification of resin exuded after 4 hours in approximately 9 year-old trees not previously tapped. Standard errors are indicated on top of bars. Asterisks indicate significant difference according to Student's t-test, with $P \leq 0.001$.

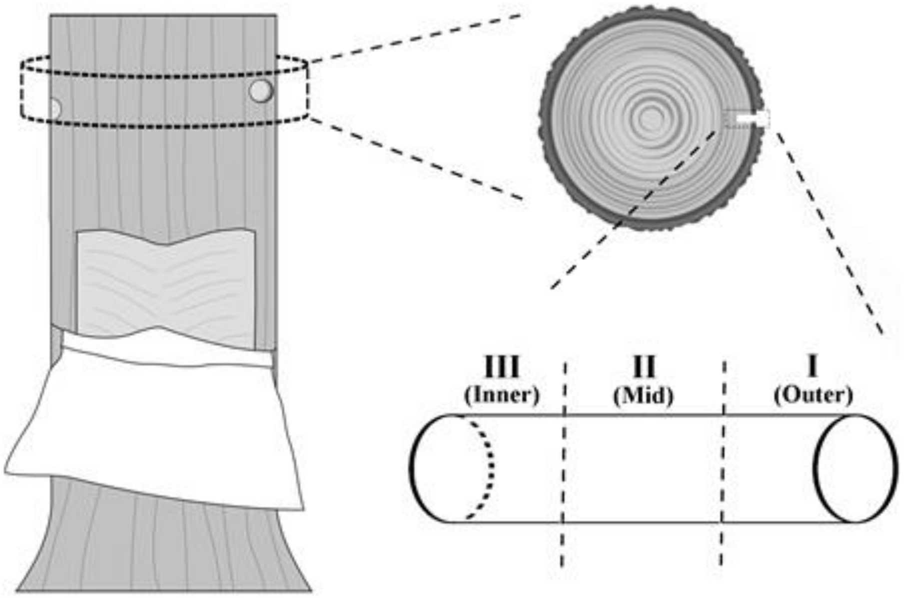

Fig. S4. Schematic of analysis of wood cores (divided into three parts) used for volume, area and diameter of resin canal evaluation. Three 5 mm diameter x 18 mm length wood cores were sampled from each tree.

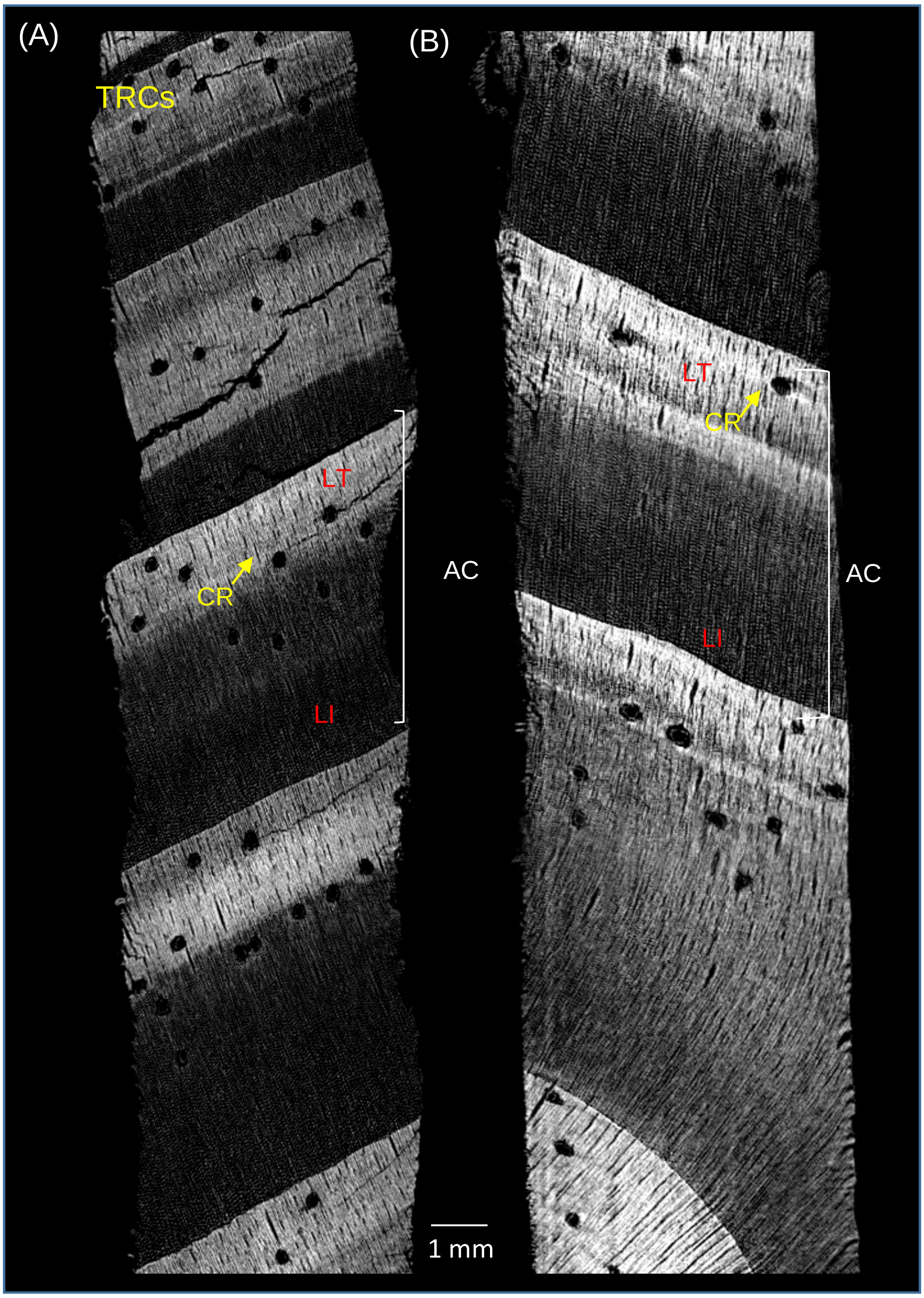

Fig. S5. Example X-ray micro-CT image of wood cores. Cross-sections: (A) trees of high resin yield and (B) low resin yield, indicating the growth ring (AC) earlywood (LI) and latewood (LT), with the resin canals (RC) e traumatic resin canals (TRCs).

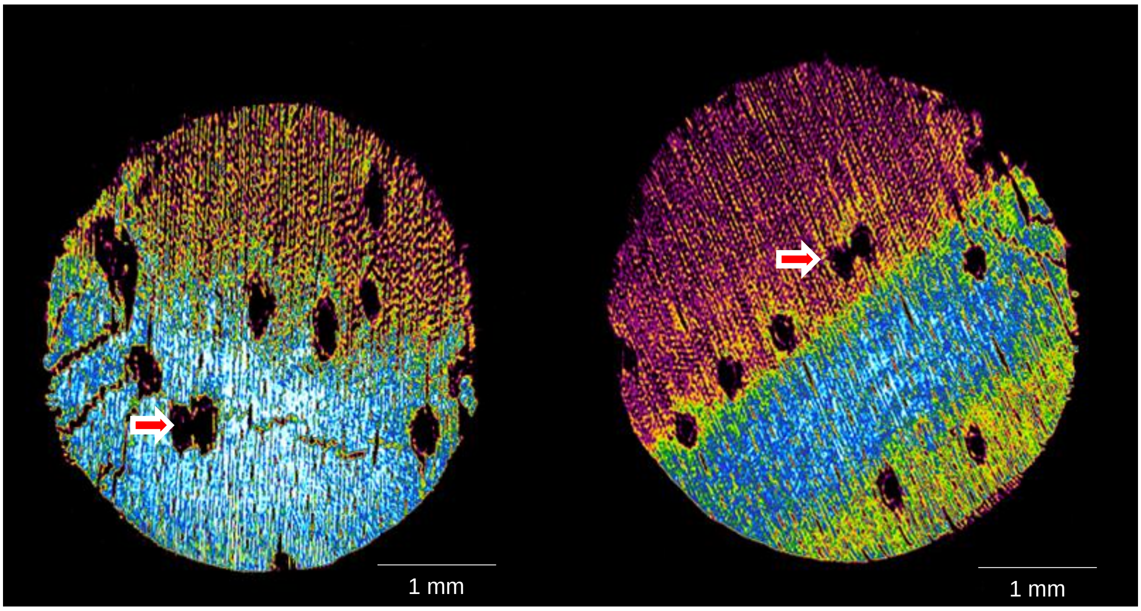

Fig. S6. Anatomical structure of the transverse plane of wood cores. High resin yield trees showing anastomosed canals (arrow). Earlywood (dark coloured in image, lower density); latewood (light coloured, higher density).

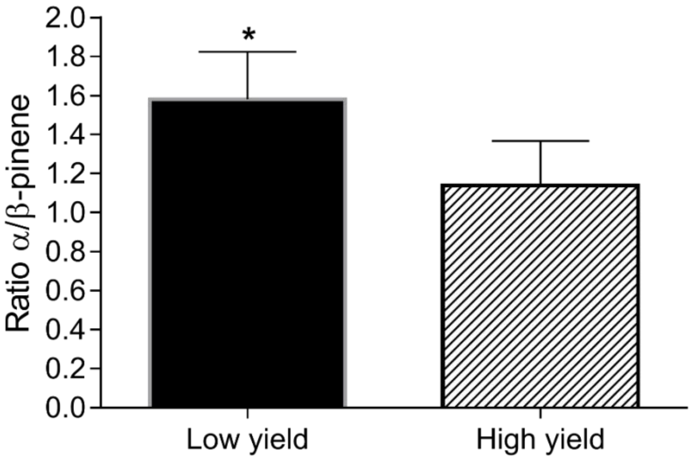

Fig. S7. Ratio α/β-pinene in low and high yield resin trees of *Pinus elliottii* (approximately 18-years-old). Standard errors of the means are indicated on top of bars. Asterisks indicate significant difference according to Student's t-test, with *P ≤ 0.001.

PRINCIPAIS RESULTADOS E PERSPECTIVAS

Os resultados apresentados nesta tese contribuem para um maior entendimento da resinose em *Pinus elliotii* e sua exploração racional mais eficaz. Foi possível observar que ambos os tipos de floresta (plantada e regenerada) tiveram rendimentos similares, com picos na primavera e no verão. As novas pastas testadas com base em ácido benzoico ou NAA, em particular a primeira, foram eficazes como estimulantes de resina, enquanto a combinação de adjuvantes de diferentes vias de sinalização não mostrou nenhum efeito sinérgico ou aditivo significativo, possivelmente, devido ao compartilhamento de moléculas de sinalização. O menor custo dos novos adjuvantes identificados em relação ao CEPA (precursor de etileno), bem como a equivalência de florestas plantadas e regeneradas para produção de biomassa de resina, pode ter implicações práticas úteis para o setor florestal e resinífero.

Atualmente, um dos principais problemas da exploração comercial da resinagem em *Pinus* é a falta de uniformidade de produção por conta da alta variabilidade genética das árvores. Além disso, o melhoramento genético de espécies de *Pinus* e sua propagação clonal são processos demorados e/ou tecnicamente limitados. Consequentemente, a exploração de resina em florestas de *Pinus* permanece muito abaixo da potencialidade genética dos indivíduos com alto perfil produtivo. Há grande interesse em se estabelecer florestas mais homogêneas e com fenótipo superresinosos para este setor florestal.

Neste trabalho foi desenvolvido e validado um método simples e rápido de identificação relativamente precoce de genótipos com rendimentos de resina superiores à média de produção de uma população de árvores resinosas, envolvendo análise rápida cinética-volumétrica de exsudação após punção, com vistas ao estabelecimento de florestas com produção superior de resina. Desse modo, as empresas que atuam no setor de resinagem podem fazer uso dessa ferramenta para minimizar o problema da heterogeneidade genética de árvores e produtividade média de resina aquém do potencial nas florestas em utilização. A partir dos indivíduos selecionados, será também buscado o aprimoramento de métodos para a propagação clonal (principalmente *ex-vitro*), inclusive por braquiblastos, de genótipos elite voltados à resinagem.

ANEXOS

ANEXO 1

Biomass yield of resin in adult *Pinus elliottii* Engelm. trees is differentially regulated by environmental factors and biochemical effectors

Franciele Antônia Neis, Fernanda de Costa, Thanise Nogueira Füller, Júlio César de Lima, Kelly Cristine da Silva Rodrigues-Corrêa, Janette Palma Fett, Arthur Germano Fett-Neto.

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ABSTRACT

Biomass of pine resin finds several applications in the chemical, pharmaceutical, biofuel and food industries. Resin exudation after injury is a key defense response in Pinaceae since this complex mixture of terpenes has insecticidal, antimicrobial and wound repair properties. Resin yield is increased by effectors applied on the wound area, including phytohormones and metal cofactors of terpene synthases. The interaction of resinosis mechanism effectors is not fully understood, particularly in adult forest setups under natural environmental variations. The aim of this work was to determine how resin exudation by wounded trunks of adult *P. elliottii* responded to combined chemical effectors involved in different regulatory pathways of resinosis (metal cofactors of terpene synthases, benzoic acid and plant growth regulators) and whether seasonal and tree distribution variations affected these responses. Symmetrically planted and scattered trees regenerated from the seed bank had similar resin biomass yields, suggesting that the homogeneity in development and spatial arrangement were not significant factors in resin yield. This new finding is of practical importance with the used tapping system since costs of implanting forests by regeneration can be advantageous compared to planting. In addition, it was shown for the first time that the salicylic acid precursor benzoic acid and the auxin naphthalene acetic acid promoted resin exudation when individually applied to wound sites. Both these adjuvants are two orders of magnitude less costly compared to the conventionally used ethylene precursors, besides facing less environmental and health restrictions for use. Most adjuvant-treated trees showed higher resin flow in the second year, indicating mechanisms of response build up. Overall, temperature was more important than rainfall as environmental parameter affecting resin biosynthesis, which was higher in the warmer months of spring and summer. The combination of resinosis stimulant effectors from different signaling pathways showed no significant synergistic or additive effect, suggesting possible converging signaling pathways and/or limitation of common intermediate transducing molecules.

1. Introduction

Pines occupy highly diverse environments, over a range of temperatures, water and nutrient availabilities, irradiance levels and photoperiods, being able to effectively face attacks from diverse herbivore and pathogen guilds. The success of conifers is linked to their complex terpene biochemistry hosted by specialized secretory cells. The terpenoid resin synthesized by *Pinus* spp. is one of the main mechanisms of defense of these trees, particularly against bark beetles and the fungi they carry (Fett-Neto and Rodrigues-Corrêa, 2012). Pine resin biomass is essentially composed of a monoterpene and sesquiterpene-rich turpentine and diterpenoid-rich rosin fraction, both finding numerous industrial applications as non-wood forest products (Rodrigues-Corrêa et al., 2012).

Molecules capable of modulating different signaling pathways have been identified as resin yield stimulators, including sulfuric acid (extends wound damage), 2-chloroethylphosphonic acid (CEPA, a synthetic ethylene precursor), paraquat (free radical generator), yeast extract (mimics attack by pathogens), salicylic acid (pathogen signaling molecule), auxin (promotes ethylene biosynthesis and resin canal differentiation), jasmonic acid (signals mechanical damage and promotes secondary metabolism) and metal ions, such as potassium, iron and manganese (cofactors of terpene synthases in conifers) and copper (a component of ethylene receptors) (Clements, 1970; Conrath et al., 2002; Fett-Neto and Rodrigues-Corrêa, 2012; Hudgins and Franceschi, 2004; Lewinsohn et al., 1994; Martin et al., 2002; Popp et al., 1995;

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Table 1
Resinosis effectors, concentrations and functional rationale.

Chemical adjuvant	Concentration	Activity	Recent source
Potassium sulfate (K_2SO_4)	500 mM	K: activation of terpene synthases	Rodrigues et al. (2011), Savage et al. (1994)
Copper sulfate ($CuSO_4$)	10 mM	Cu: part of ethylene receptor	Rodrigues et al. (2011), Rodriguez et al. (1999)
Naphthaleneacetic acid	1 mM	increases ethylene biosynthesis and resin canal differentiation	Chae and Kieber (2005), Fahn (1982)
Benzoic acid	10 mM	phytoalexin and precursor of salicylic acid, a defense phytohormone	Franich et al. (1986), Sha (2003)
Chloroethylphosphonic acid (CEPA)	3% (w/v)	releases ethylene, a defense-related phyhormone	Chae and Kieber (2005)

Rodrigues et al., 2011; Rodrigues et al., 2008; Rodrigues and Fett-Neto, 2009; Stubbs et al., 1984). Research efforts aiming at finding lower cost resin stimulant adjuvants with equivalent or superior performance of CEPA (most used currently) are of interest to the tapping industry. Although synergistic responses in wound-related resin production by supplying combined metal cofactors of terpene synthases were not observed (Rodrigues-Corrêa and Fett-Neto, 2013), simultaneous treatments with effectors acting in different signaling pathways (for example, metal ions and auxin) have not been previously examined. In addition, it is not clear how wound-related resin biosynthesis and accumulation under field conditions in adult trees of subtropical introduced slash pine plantations is affected by climate factors, namely temperature and precipitation.

To better understand the roles of phytohormones and some of their precursors, receptor components and metal cofactors of terpene biosynthetic enzymes on wound response defense resin flow in relation to climate, this study investigated the following hypotheses: a) resin biomass stimulators involved in different signaling response pathways are more effective combined than alone; b) stimulant effect of resin flow stimulators is season dependent; c) temperature is more significant in modulating resin production than rainfall. The study examined adult 16-year-old slash pine forests derived from planted seedlings or regenerated from the seed bank, submitted to one of 9 treatments, for 2 years, and involved over 1600 individually tracked trees.

2. Materials and methods

2.1. Plant material, wounding procedure, resin harvest and quantification

Slash pine (*P. elliottii* Engelm. var. elliottii) trees (16 years old), grown in two forest stands in northeastern Rio Grande do Sul, city of Balneário Pinhal (approximately 30.19° south latitude and 50.36° west longitude) were used in the experiments. The forests of similar genetic composition were in the installations of Celulose Irani S.A. One of the stands was established by regeneration of individuals from the seed bank previously deposited by the last felled forest, whereas the second tree stand originated from planted seedlings. The effect of different individual or combined effectors that operate in diverse physiological pathways (benzoic acid, plant growth regulators, co-factor metals of terpene synthases) was evaluated on resinosis. Potential synergism between pathways on resin production was examined. Randomly distributed trees (a total of 1620, being 900 trees in the regenerated forest and 720 trees in the planted forest) with circumference at breast height ranging from 65 to 90 cm were used. Trees were evaluated for two years and the experiments started in winter (2013). All trees used in the study had not been damaged or explored for resin prior to the experiments and shared the same previous history, except for the mode of stand establishment (regenerated or planted). A total of 80 and 100 trees from planted and regenerated forest, respectively, were used in each treatment.

Bark strips approximately 2.5 cm wide and 2–5 mm high were removed biweekly with a bark shaving tool, corresponding to approximately one-third of the tree circumference, exposing the sapwood surface. Seasonal resin production was evaluated for a period of two years (2013–2015). At the end of each season (winter, June–August; spring, September–November; summer, December–February; fall, March–May, approximately 90 days each), accumulated resin in plastic bags belted under the wounding panel was harvested and weighed on a field digital balance after careful removal of the upper layer of rainwater, which minimized resin volatile loss during the experiments (Rodrigues et al., 2008). Plastic bags were regularly checked and replaced as required to avoid any resin leak.

2.2. Treatments

Nine treatments were evaluated in each forest type. Pastes produced with different chemical effectors alone or in combination were applied on the wound panel right after each bark stripping (Table 1).

Each treatment paste contained 20% sulfuric acid in aqueous solution as basal active constituent and rice husk powder as an inert substrate to optimize paste consistency and residence time on the wound line (Fuller et al., 2016). Single modifications in the composition of the resinosis paste were done by including: K (500 mM potassium sulfate); BA (10 mM benzoic acid); Cu (10 mM copper sulfate); NAA (1 mM Naphthaleneacetic acid); K + BA (500 mM potassium sulfate + 10 mM benzoic acid); K + BA + Cu (500 mM potassium sulfate + 10 mM benzoic acid + 10 mM copper sulfate); K + BA + Cu + NAA (500 mM potassium sulfate + 10 mM benzoic acid + 10 mM copper sulfate + 1 mM naphthaleneacetic acid); CEPA (3% 2-chloroethylphosphonic acid, which is the most frequently used resinosis effector, herein used as a positive control), negative control (only streaking without paste application).

2.3. Resin composition analysis

Resin samples were taken from the trunks of five randomly selected trees belonging to each of the following treatments: potassium sulfate, NAA, CEPA or only bark streak (as described on section 2.2). Each sample consisted of a mixture of equal proportions of freshly flowing resin. The trees used in this study had been previously tapped for resin production for one year with the respective stimulant paste. Samples were collected in the fall of 2014. Immediately after harvest, all samples were frozen in liquid nitrogen and kept as such until storage at −80 °C. The preparation of samples for terpene analysis was performed as previously described (Wang et al., 1997). Extractions and analyses were done in triplicate. Quantitative and qualitative analyses of resin were done by gas chromatography (GC) and GC-Mass Spectrometry, respectively, as previously described (Rodrigues et al., 2011).

2.4. Weather and soil data

Weather data (temperature and rainfall) were obtained from the nearest meteorological station, located 40.5 km from the forest (city of Tramandaí). Potential Evapotranspiration (PET) was calculated using the Thornthwaite monthly water-balance program (McCabe and Markstrom, 2007). The soil in the area is classified as Hydromorphyc Planosoil, Eutrophic Solodic (Streck et al., 2008). No fertilization was applied throughout the study.

2.5. *Statistical analyses*

Experimental set up was completely randomized. Analyses of variance (ANOVA) followed by Tukey test when appropriate were used for data analysis. Data were log transformed when necessary to fit the ANOVA requirements of variance homogeneity. Student's *t*-test was used for simple comparisons of two data sets. Differences were considered significant for $P \leq 0.05$. Data were expressed as mean ± standard error (S.E.). Correlations between environmental parameters (temperature and precipitation) and resin yield in each one of the years evaluated were analyzed by Pearson Correlation Coefficient. Overall linear regression between temperature and resin yield data was calculated using GraphPad Prism 6.0 (Prism, 2014).

3. Results

The trees used in all treatments and in both forest stands remained fully viable, and did not show any visible signs of loss of vigor, disease or decaying problems. Mean annual rainfall in the study area was 443 ± 43 mm (469 year 1 and 425 year 2). Overall higher water availability in the first year was confirmed by the Thornthwaite model of potential evapotranspiration (Supplementary information, Fig. S1). Seasonal minimum and maximum temperatures ranged from 13.9 to 24.3 °C and 14.7 to 25.3 °C, for the first and second year, respectively (Fig. 1A).

There was a clear seasonal pattern of resin yield in both stands. Mean seasonal resin biomass was higher in summer in the first year and spring in the second year (1.35 and 1.36 kg, respectively) (Fig. 1B). Resin production during winter season increased approximately 72% in the second year compared to the same season in the first year (Fig. 1B). This result was coincident with changes observed in weather data. Rainfall varied considerably between the first and second years, except during spring (Fig. 1C). During winter and summer, there was a significant decrease in precipitation in the second year when compared to the year before. Precipitation in the second year was almost twice as high as that of the first during fall (Fig. 1C); however, resin yield in this season was similar in both years (Fig. 1B). Average temperature was similar in summer and fall for both years evaluated (Fig. 1A). However, during winter and spring the mean temperature was slightly higher in the second year.

Temperature rather than precipitation appeared to be the environmental factor more closely associated with resin yield within the observed period. Significant positive correlation was found between resin yield and temperature in both first ($r = 0.95$, $P < 0.01$) and second year ($r = 0.78$, $P < 0.01$). Precipitation correlation with resin production was negative ($r = -0.41$ and $r = -0.90$ for first year and second year, respectively), but did not correlate significantly with resin yield considering a minimum probability of 5%. Overall linear regression between temperature and resin yield was highly significant ($R^2 = 0.76$, $P = 0.005$) (Supplementary information, Fig. S2).

In both stands, there was an increase of approximately 15% in the basal resin biomass yield (bark streak only) during the second year. Trees treated with paste containing CEPA produced higher amounts of resin (5.1 kg) when compared to other pastes during the first year (Fig. 2). In the second year, trees treated with paste containing BA, Cu or BA combined with other effectors had the same resin yield of trees exposed to CEPA (Fig. 2). Resin yield of trees not treated with effectors (negative control) was consistently lower than that of treated trees (2.6 kg in the first year and 3.0 kg in the second year), showing their positive impact on resinosis (Fig. 2).

Significant effects of stand establishment method were not observed. Trees of planted forests had the same resin yield (4.5 kg/tree) when compared to regenerated forest trees (4.4 kg/tree), considering overall analysis of all treatments during both years, the same applying to seasonal production (Supplementary information, Fig. S3).

Significant differences in resin yields between treatments were observed. In general, CEPA treatment promoted higher yields of resin along the annual evaluation compared to other treatments, followed by BA (Fig. 3). Seasonal differences in resin yields were noticed. Higher resin production was recorded during the summer and spring, mostly in the former. Effector performances also showed seasonal response. CEPA was beneficial for winter yield (Fig. 3). The metals K and Cu were effective stimulators of resin exudation and yielded equivalent amounts relative to CEPA treatment in summer (K and Cu), spring (Cu), and fall (K) (Fig. 3). BA proved to be a positive effector of resinosis, resulting in yields similar to those of CEPA treated trees in all seasons but winter (Fig. 3). NAA was also an active resinosis effector compared to control trees in all seasons and equivalent to CEPA-treated trees in the summer (Fig. 3).

The main monoterpene constituents in resin derived from control and effector-treated trees were α-pinene and β-pinene, followed by limonene and camphene (Table 2). The overall proportion of α-pinene, camphene and limonene was not significantly affected within the different effector treatments. However, the concentration of β-pinene increased in resin of trees treated with NAA, ranging from approximately 38.5% in control trees to almost 48% of total monoterpenes in the resin

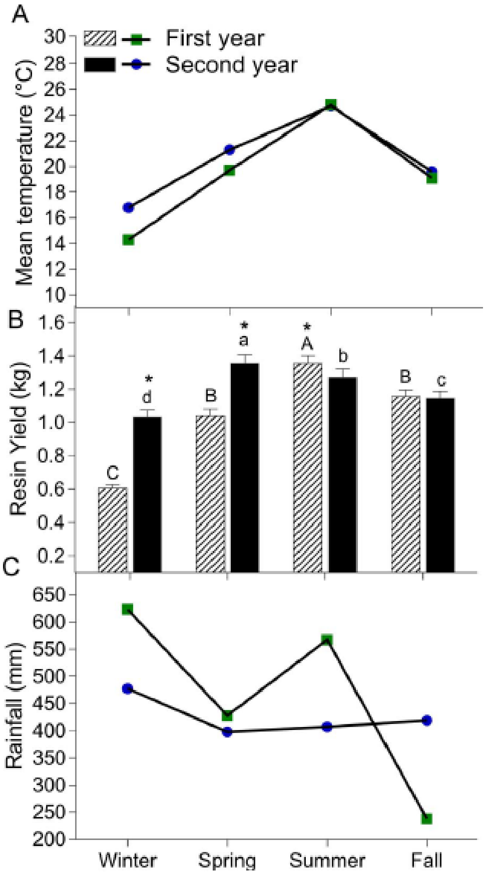

Fig 1. Environmental data and production of resin in pine trees treated with different resinosis effectors. (A) mean temperature (B) resin yield per season represented by dashed bars (first year) and black bars (second year). Standard errors of the means are indicated on top of bars. Different letters indicate significant difference among seasons within the same year by Tukey test ($P \leq 0.05$). Asterisks indicate significant differences between the years within each season, according to the Student *t*-test ($P \leq 0.001$). (C) mean rainfall. In (A) and (C), square symbols refer to first year and circles to second year data.

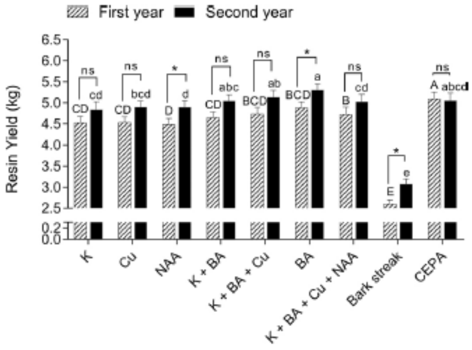

Fig. 2. Annual production of resin. Bars represent annual yield of resin per treatment indicated in the first (dashed) and second (black) year. K = potassium; Cu = copper; NAA = naphthaleneacetic acid; BA = benzoic acid; CEPA =2- chloroethylphosphonic acid. Standard errors of the means are indicated on top of bars. Different letters indicate significant difference among treatments within the same year by Tukey test ($P \leq 0.05$). Asterisks indicate significant differences between the years within each treatment, according to the Student *t*-test ($P \leq 0.001$).

Table 2
Concentration (as% of total terpenes) of monoterpenes in *Pinus elliottii* var. elliottii resin. Numbers within the same column sharing a letter do not differ by a Tukey test ($P \leq 0.05$).

Treatment	α-pinene	β-pinene	camphene	limonene
K	49.191^a	36.752^b	0.353^a	13.704^a
NAA	39.391^a	47.902^a	0.475^a	12.232^a
Bark streak	48.588^a	38.518^b	0.550^a	12.344^a
CEPA	42.587^a	40.118^b	0.481^a	16.815^a

of trees exposed to NAA (Table 2).

4. Discussion

Resin yield was significantly affected by tapping season. In the first year, relatively lower amounts of resin were observed during winter coinciding with low temperatures and high precipitation. However, in the second year, there was an increase in yield, possibly as a result of higher average temperature and decreased precipitation (Supplementary information, Fig. S2 and Fig. S3) when compared to the first year (Fig. 1). Rodríguez-García et al. (2014) observed higher resin yield in soils with more water retention. Low temperatures normally lead to a depression in resin production (Brito et al., 1978), so the increase in temperature during winter was coincident with relatively higher resin yield. In subtropical climates, in which winters are mild, resin tapping can be carried out year-round (Rodrigues and Fett-Neto, 2009). The higher yields in the second year could also be the result of

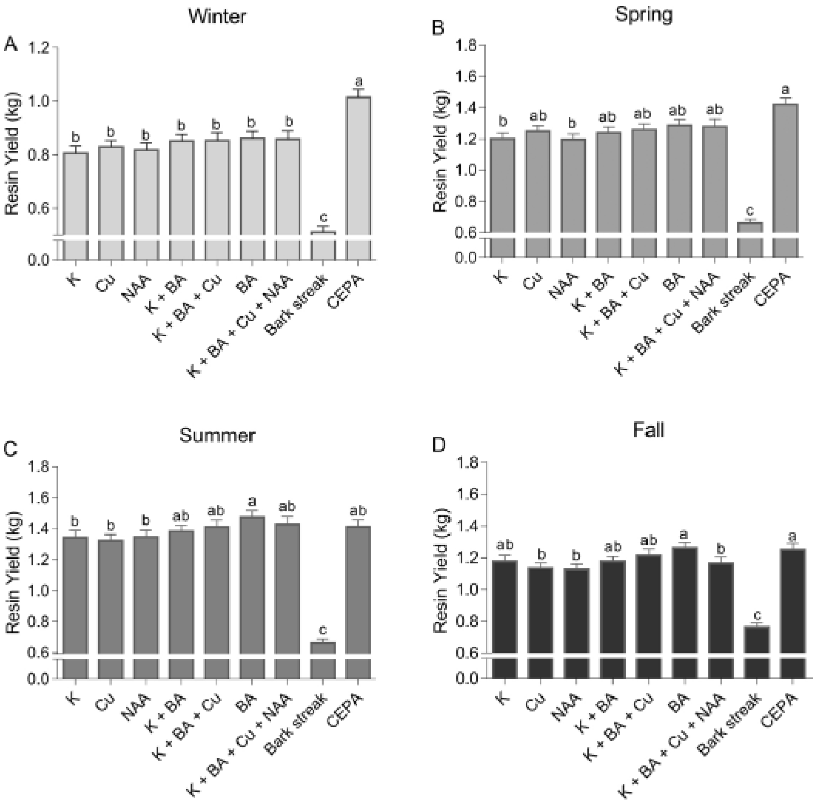

Fig. 3. Mean resin yield of two years: combined effects of season and chemical effectors. K = potassium; Cu = copper; NAA = naphthaleneacetic acid; BA = benzoic acid; CEPA =
chloroethylphosphonic acid. Bars sharing a letter are not significantly different by Tukey test ($P \leq 0.05$) within a particular season. Standard errors of the means are indicated on top
bars.

increased development of resin canals due to systemic wounding response, as observed in *Pinus pinaster* (Rodríguez-García et al., 2014).

The high resin yield in summer and spring may be due to favorable environmental conditions of temperature and precipitation compared to the annual average. Previous experiments carried out in subtropical climate of southern Rio Grande do Sul revealed spring as the most productive season for resin tapping (Rodrigues and Fett-Neto, 2009).

In spite of the relative water stress condition during the first year of production, fall resin yield was similar in both years (Fig. 1). This profile differs from *Pinus ponderosa*, in which resin biomass increased later in the growing season under moderate water stress. In this condition, carbon demand for growth decreases but photosynthesis continues, presumably causing a shift of carbohydrate allocation from growth to defense (resin production) (Hood and Sala, 2015). Since fall temperatures were very close in both years, for slash pine it appears that temperature rather than precipitation was a major environmental determinant of resin yield at least in the present study conditions. This observation is also somewhat supported by the results of spring yield in both years (Fig. 1). Indeed, there was a significant positive correlation between temperature and resin yield in both years.

The fact that the mean overall yield of planted and regenerated forests was not significantly different indicates that relative heterogeneity in developmental stage and spatial arrangement layout of the trees are not critical factors in resin yield, at least under the conditions examined. This fact has practical significance in the establishment and management of forest stands to be explored for resin with the used tapping system or for regeneration of disturbed areas. Cost of stand implementation may be reduced by managing forest regeneration from the seed bank (Duryea, 1987).

Higher resinosis of effector-treated trees were recorded in the second year compared to the first (Fig. 1). This response could be a function of delays in a number of processes in the first year, such as even distribution of effectors, triggering of responsive pathways and/or wound induced duct differentiation.

CEPA promoted resin flow, especially in winter (Fig. 3). CEPA releases ethylene, which may trigger stimulation of resin synthesis in existing resin ducts, and also induce the formation of traumatic ducts (Sharma and Lekha, 2013). Under subtropical conditions of southern Brazil, higher resin yields from *P. elliottii* have been obtained by using stimulant pastes containing sulfuric acid and CEPA as active components (Rodrigues et al., 2008).

The replacement of CEPA with BA proved to be effective in the second year of application. BA is a precursor of the plant hormone salicylic acid (SA), one of the key endogenous signals involved in defense response activation (Chen et al., 2009; Shah, 2003). SA is an important signaling molecule for eliciting responses to several abiotic and biotic stresses (Khan et al., 2012; Miura and Tada, 2014). The attack by pathogens or treatment of plants with synthetic compounds can induce and/or promote SA signaling, promoting more efficient activation of the defense response (Bektas and Eulgem, 2014; Conrath, 2009; Slaughter et al., 2012). It is possible that exogenous application of BA could activate defense responses and modulate resin production by increasing SA levels (Chen et al., 2009). BA itself has also been regarded as a defense phytoalexin in *P. radiata* (Franich et al., 1986), which may have contributed to the deployment of resin flow in slash pine.

NAA proved to be a resinosis effector. The efficiency of auxin in stimulant pastes in subtropical climates had been previously shown with 2,4-dichlorophenoxyacetic acid (2,4-D), even as a replacement for CEPA, particularly in the second year (Rodrigues et al., 2008). Auxin action may stimulate ethylene production and promote resin canal differentiation (Rodrigues et al., 2008). NAA is advantageous over 2,4-D because it faces less environmental restrictions for use in certified forests. Interestingly, NAA treatment increased the concentration of β-pinene, which is relevant for defense capacity of resin exudate and for resin industrial uses (Arango-Velez et al., 2016; Rodrigues et al., 2011), suggesting monoterpene synthase-specific stimulation, possibly at gene expression level (De Lima et al., 2016).

Although the direct contribution of paste adjuvants in the overall cost of tapping operations is relatively small, the cost of NAA and BA are two to three orders of magnitude lower than that of CEPA (see for reagent grade values Sigma-Aldrich, 2017). Moreover, potential environmental damage, workers' health and safety issues for BA are less severe compared to those of CEPA (Sigma-Aldrich, 2017).

In the present study, trees were approximately 16 years old. Field tests with 28-year-old slash pine in the southern shore of Rio Grande do Sul (approximately 400 km further south of the sites used in the current experiments) showed that potassium treatment led to superior yields of resin along a four-year evaluation compared to commercial paste (Rodrigues-Corrêa and Fett-Neto, 2013). In the present tests, seasonal and area related responses were observed when using potassium-containing paste. Nonetheless, the use of potassium promoted resin yields throughout the two-year evaluation, especially in the spring, summer and fall in the regenerated forest. These differences in response profile may reflect age, variations in genetic composition of founding plant material, number of years evaluated, and environmental-related factors. In fact, positive relationship between resin yield and tree age was observed by Tolera et al. (2015), who investigated frankincense yield variation in *Boswellia papyrifera*.

The combination of effectors of different physiological and signaling pathways did not show overt synergic or additive effects in resin production. This is in agreement with previous results combining metal cofactors, which was attributed to problems in their distribution in the tree and competition for transporters or binding sites in target secretory cells of resin canals (Rodrigues et al., 2011). Abscisic acid transport has been pointed out as a limiting factor for more efficient environmental stress signal intensity transduction mediated by this phytohormone in rice (Ye et al., 2012). The current lack of potentiated effects of combined effectors involved in different signaling routes on resin yield may be the result of crosstalk among pathways, so that common transduction components could become limiting for further activation (Hansen and O'Shea, 2015). Future investigations could address using alternate paste adjuvants depending on the season (*e.g.* BA in warmer seasons and CEPA in winter), as well as new ratios of these adjuvants in more even concentrations.

5. Conclusions

In spite of acting in distinct signaling biochemical pathways, resinosis effectors did not show synergism or additive interactions, being equally efficient when applied alone. Resin production effectors had season dependent impacts on resinosis, being overall more efficient stimulants in warmer months. Temperature modulated resin flow to a greater extent than rainfall. Benzoic acid and NAA applied alone, notably the former, were effective resinosis stimulators in slash pine. From a practical perspective, these newly described regulatory features of the physiology of slash pine resinosis favor resin production, supporting the use of simpler and less costly stimulant pastes, as well as managed regenerated forests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.indcrop.2018.03.027.

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SUPPLEMENTARY FIGURES

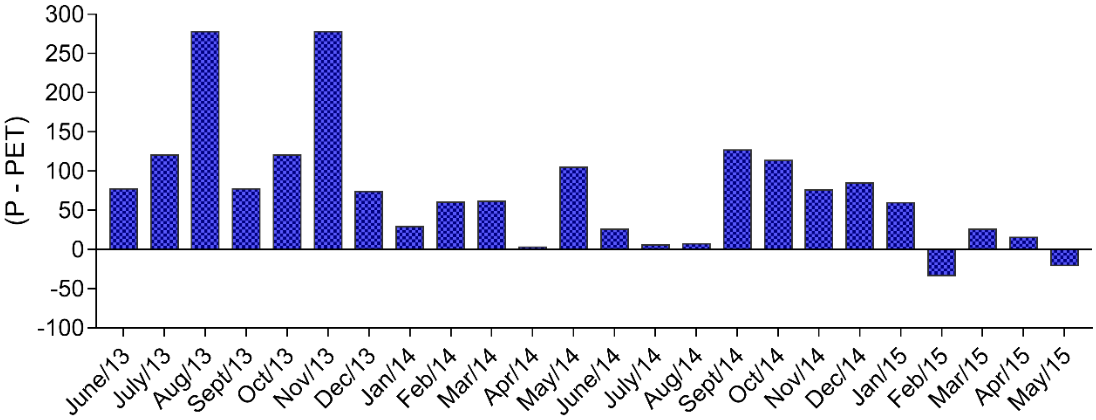


Fig. S1. Monthly precipitation during the studied period. Legend: P, monthly accumulative precipitation (mm); PET, Thornthwaite potential evapotranspiration (mm).

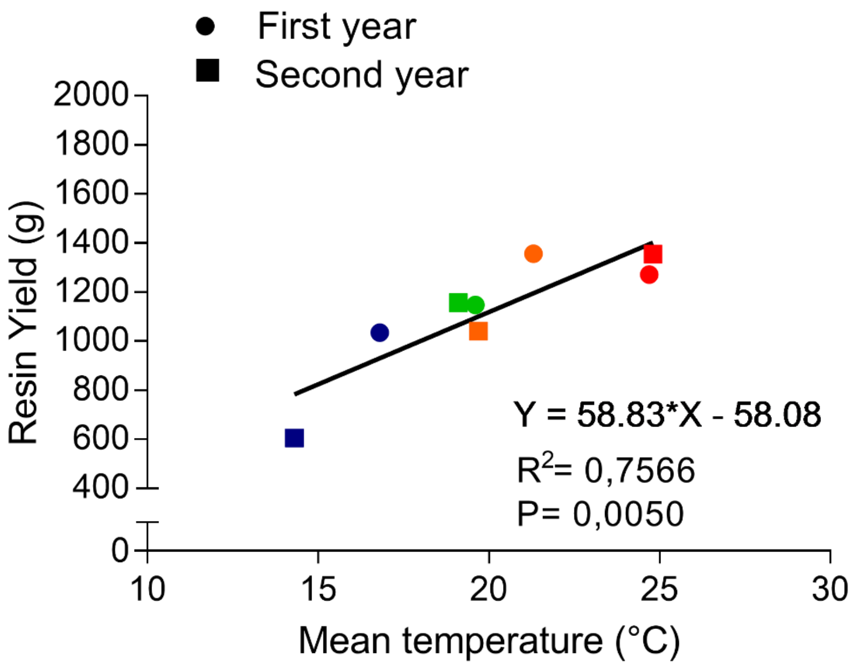


Fig. S2. Correlation between resin yield and average temperature for two years of evaluation. Square symbols refer to first year and circles, to second year data. Colors represent seasons: blue (winter), green (fall), orange (spring) and red (summer). Linear regression was used to estimate R^2 = 0.7566 and P = 0.005.

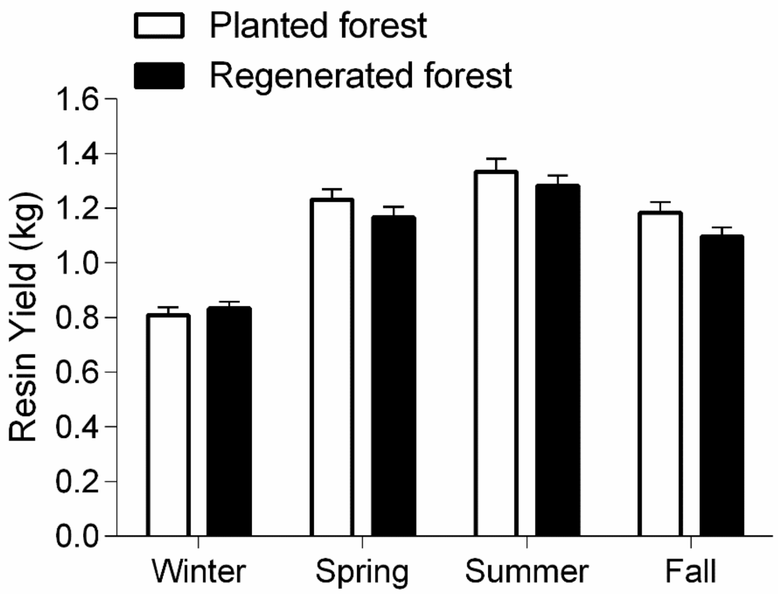


Fig. S3. Seasonal mean resin yield of two years in trees from planted and seed bank regenerated forests. There are no significant differences between seasons, according to the Student *t*-test, with $P \leq 0.05$. Lines on top of bars represent standard error of the means.

ANEXO 2

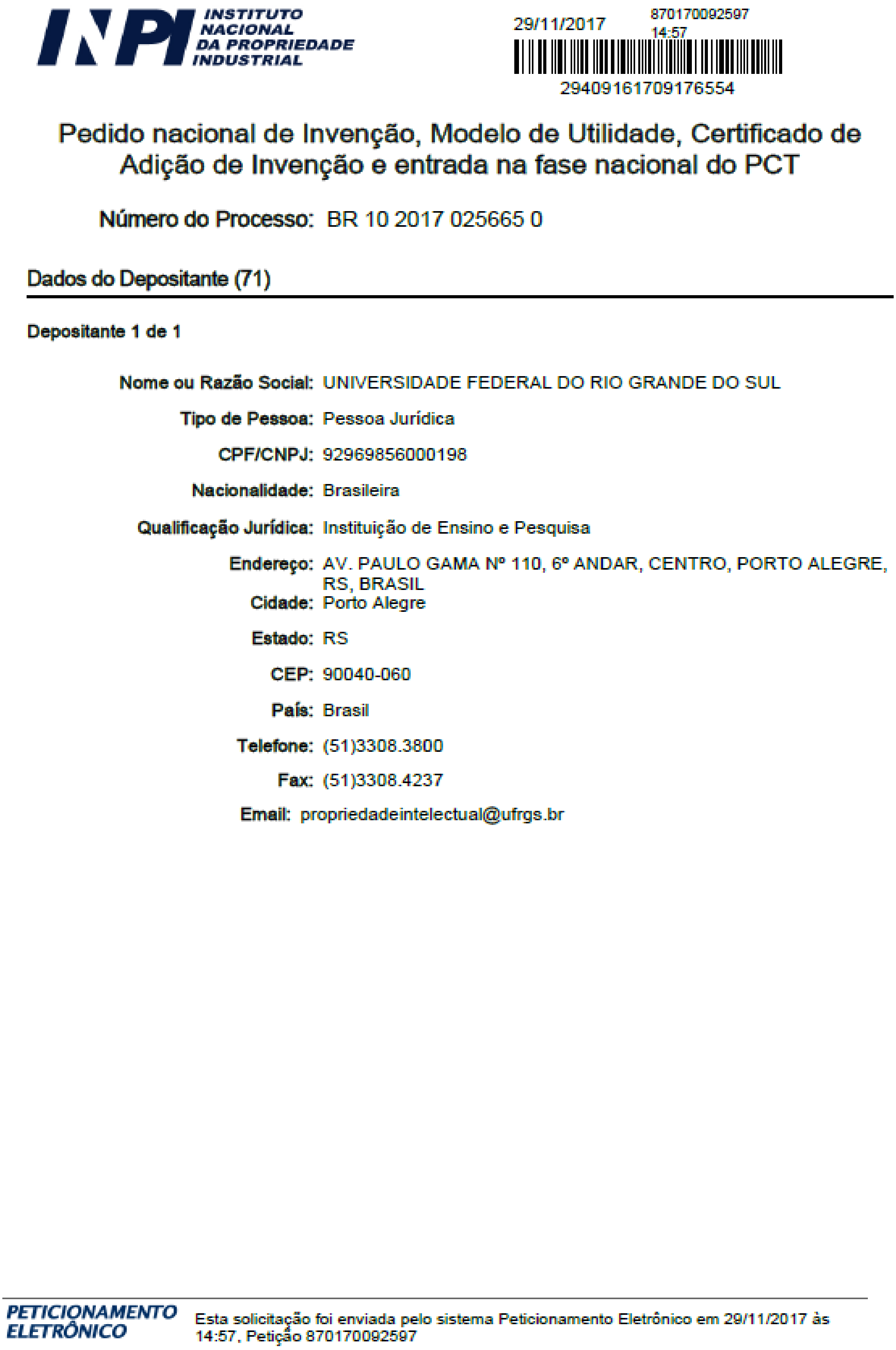
Patente de Invenção (PI)

Título da Invenção/ Modelo de Utilidade: Método de identificação precoce de genótipo com alta produção de resina e método para estabelecer florestas resinosas compostas de genótipos superresinosos e homogêneas para resinagem.

Inventores:

ARTHUR GERMANO FETT NETO

FRANCIELE ANTÔNIA NEIS



INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

29/11/2017
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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 025665 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 92969856000198
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
Endereço: AV. PAULO GAMA Nº 110, 6º ANDAR, CENTRO, PORTO ALEGRE, RS, BRASIL
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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 29/11/2017 às 14:57, Petição 870170092597

ANEXO 3

Curriculum vitae

DADOS PESSOAIS:

Nome: Franciele Antônia Neis

Filiação: Miguel Neis e Maria Gessi Neis

Nascimento: 27/01/1988 – Caiçara, RS – Brasil

Endereço eletrônico: franci_neis@yahoo.com.br

FORMAÇÃO ACADÊMICA/ TITULAÇÃO

2014 -: Doutorado em Biologia Celular e Molecular – Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Título: Aspectos Fisiológicos e Anatômicos da Resinose em *Pinus elliotti* Engelm.

Orientador: Arthur Germano Fett Neto

Co-orientadora: Janette Palma Fett

Bolsista: CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2011-2013: Mestrado em Agrobiologia – Universidade Federal de Santa Maria, UFSM, Santa Maria, Brasil

Título: Efeito do cobre na produção de β-ecdisona em *Pfaffia glomerata* (Spreng.). Pedersen

Orientador: Fernando Teixeira Nicoloso

Bolsista: Pioneer Hi-Bred International Research

2006-2010: Graduação em Ciências Biológicas – Universidade Regional Integrada do Alto Uruguai e das Missões (URI)

Título: Levantamento das Áreas de Preservação Permanente em um trecho adjacente a Linha Boa Vista do Pardo na Bacia do Rio Uruguai no Município de Caiçara - RS

Orientador: Antonio Valmor de Campos

ESTÁGIOS REALIZADOS

2010 – 2010: FATEC - Fundação de Apoio à Tecnologia e Ciência - Santa Maria
Projeto: Manejo de água em sistemas Agrícolas (210h).
Orientador: Reimar Carlesso

ATUAÇÃO PROFISSIONAL

2013-2015: Professora substituta - Universidade Federal de Santa Maria (UFSM), 40h.

Disciplinas ministradas:

- Ecologia dos Ecossistemas
- Ecologia geral
- Princípios de Sistemática Biológica
- Sistemática de Algas e Fungos
- Sistemática de Arquegoniadas e Gimnospermas
- Sistemática das Magnoliophyta
- Botânica
- Paleontologia
- Biogeografia
- Biodiversidade
- Gestão Ambiental
- Estágio Curricular Supervisionado das Ciências Biológicas no Ensino Médio
- Estágio Curricular Supervisionado das Ciências Biológicas em Espaços Educativos
- Estágio Curricular Supervisionado das Ciências Biológicas no Ensino Fundamental

ARTIGOS COMPLETOS PUBLICADOS EM PERIÓDICOS

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THEISEN, G. R.; BORGES, G. M.; VIEIRA, M. F.; KONFLANZ, T. L.; NEIS, F. A.; SIQUEIRA, A. B. Implantação de uma horta medicinal e condimentar para uso da comunidade escolar. Revista Eletrônica em Gestão, Educação e Tecnologia Ambiental. v.19, p.167 - 171, 2015.

RESUMOS PUBLICADOS EM ANAIS DE CONGRESSOS

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SCHAICH, G; SAUSEN, D.; NEIS, F. A.; ULIANA, S. C; SORIANI, H. H.; FARIAS, J. G.; HILGERT, M. N; FARIAS, A.; SASSO, V.; NICOLOSO, F. T. Relação de respostas fisiológicas de batata com a qualidade na avaliação da eficiência de uso e resposta ao fosfóro In: XXIX Reunion Argentina de Fisiologia vegetal, 2012, Mar Del Plata. La producción de alimentos y la Fisiología Vegetal: Buenos Aires: Orientación Gráfica Editora SRL, 2012.

SAUSEN, D.; NEIS, F. A.; ULIANA, S. C; FARIAS, J. G.; SORIANI, H. H.; ROSSATO, L. V.; NICOLOSO, F. T. Resposta de genótipos de batata cultivadas *In Vitro*, submetidas a doses de fósforo In: XXIX Reunion Argentina de Fisiologia vegetal, 2012, Mar Del Plata. La producción de alimentos y la Fisiología Vegetal: "Nuevos desafíos para en mundo cambio". Buenos Aires: Orientación Gráfica Editora SRL, 2012.

SAUSEN, D.; NEIS, F. A.; ULIANA, S. C; SORIANI, H. H.; NUNES, P. A. A; HILGERT, M. N; SCHAICH, G; NICOLOSO, F. T. Resposta do crescimento de plantas de batata cultivadas *In Vitro* sob duas doses de fosfóro In: XXIX Reunion

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NICOLOSO, F. T; FARIAS, J. G.; NUNES, P. A A; SCHAICH, G; ROSSATO, L.; HILGERT, M. N; ULIANA, S. C; NEIS, F. A.; SAUSEN, D.; Dressler, V. L. Differential partitionig of arsenic in reproductive organ of five rice cultivars In: XIII Congresso Brasileiro de Fisiologia Vegetal XIV Reunião Latino-Americana de fisiologia Vegetal Mudanças Climáticas Globais: Do Gene à Planta, 2011, Búzios - Rio de Janeiro. Brazilian Journal of Plant Physiology (Impresso). Londrina - PR: Brazilian Society of Plant Physiology, 2011. v.1.

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NEIS, F. A. Avaliação Ad Hoc Projeto de Pesquisa Universidade Federal Fronteira Sul, 2015.

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PATENTES E REGISTROS

FETT-NETO, A. G.; NEIS, F. A. Método de identificação precoce de genótipo com alta produção de resina e método para estabelecer florestas resinosas compostas de genótipos superresinosos e homogêneas para resinagem. 2017. Categoria: INPI - Instituto Nacional da Propriedade Industrial. País: Brasil. Natureza: Patente de Invenção. Número do registro: BR1020170256650. Data de depósito: 29/11/2017. Depositante/Titular: Universidade Federal do Rio Grande do Sul.