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Bases Moleculares da Resinagem em *Pinus elliottii* Engelm

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Tese de Doutorado

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*Esta tese é dedicada aos meus avôs, Juventino e Darcisio (*in memoriam*). Por me ensinarem sobre o valor do caráter, humildade e educação.*

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(Peço desculpas ao leitor, mas não saberia escrever estes agradecimentos se não em forma de prosa, contando as histórias e motivos que levaram a incluir cada pessoa citada aqui...)

Quando eu tinha uns oito anos, lembro de estar conversando com meu avô enquanto ele trabalhava no seu jardim, e disse para ele em tom de brincadeira que quando eu crescesse iria ser (alguma profissão boba que não recordo, pois realmente não era importante e não estava falando sério). E ele me respondeu "Não. Minha filha vai ser doutora". Acho que para ele, uma pessoa simples e sem grande formação escolar (mas com uma sabedoria e conhecimento infinitos), o "doutora" significaria seguir uma profissão como engenharia, direito ou medicina. Mas eu levei a sério, e procurei ser uma doutora de verdade. Em parte, para honrar tudo o que aprendi com ele. Em outra, para seguir o conselho do meu outro avô, que ensinou que nada é mais importante do que o conhecimento... E que o estudo é a única coisa que ninguém pode nos tirar; que tudo o que aprendemos é nosso para sempre, nossos verdadeiramente únicos e mais valiosos bens. A estes dois senhores a quem eu tenho muito a agradecer, um deles ainda forte e me enchendo de orgulho e o outro já falecido, esta tese é dedicada.

Foram muitos passos até este momento. Durante toda minha carreia acadêmica, minha mãe não mediu esforços para que eu tivesse a formação que eu queria. Foi ela quem me apoiou incondicionalmente quando optei por uma graduação com nome impronunciável e longe de casa... mesmo quando todos a julgavam irresponsável por deixar uma "menina menor de idade" ir morar em outro estado. Ela me ensinou tudo o que sei e que sou, sem nunca ignorar ou menosprezar minha curiosidade. Se sonhei alto um dia, foi porque sabia que ela havia me dado caráter e força para conquistar qualquer coisa. Sei que ela teria feito muito mais por mim, se estivesse em suas mãos. Tenho consciência de que em muitos momentos só tive condições de continuar porque ela estava por perto e acreditava em mim, e eu não suportaria desapontá-la. Este título também é dela.

Agradeço imensamente à minha família, em especial minha tia e minha avó. Duas mulheres fortes e dedicadas, que compreenderam minhas ausências, incentivaram meus sonhos mesmo sem entender meus motivos, e me apoiaram nas minhas decisões. Tenho muita honra de tê-las comigo e de tudo o que já aprendi com ambas.

Meu namorado, que embarcou comigo nessa aventura que foi a pós-graduação, e que no fim conseguiu sobreviver e me manter sã. Ele participou ativamente de todos os momentos, em todos os artigos publicados, apresentações, disciplinas, seminários, relatórios, provas... deu suporte de diversas maneiras para mim e para meus colegas em tudo o que me comprometi a fazer pelo laboratório. Ele tornou meus dias mais fáceis e permitiu que nenhuma preocupação me atingisse quando precisava trabalhar em algo mais sério, e o fez de maneira incansável e efetiva. Este doutorado não teria existido sem ele.

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eram importantes neste sentido. Mais do que amigas, foram uma família, mesmo quando distantes.

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Meus alunos de iniciação científica João, Júlia, Juliana e Magnus, que se dispuseram a encarar os mais diversos projetos dentro do meu doutorado, me auxiliaram em todo meu aprendizado e que me ensinaram muito sobre como orientar e como aprender. Todo este trabalho dependeu demasiadamente do esforço de todos os quatro. Levarei na memória a lição destas mentes sedentas de conhecimento, com indagações pertinentes que fizeram questionar toda a minha metodologia. Apresentaram soluções fundamentais para problemas que enfrentamos, foram ágeis e competentes na avaliação dos resultados, opinaram sobre a instalação de todos os experimentos... e foram premiados pelo esforço, o que me deu muito orgulho e satisfação pelo trabalho bem feito.

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“Entretanto a ciência, ela também é imprevisível. A pesquisa é um processo sem fim sobre o qual nunca se pode dizer como evoluirá. O imprevisível está na própria natureza da empresa científica. Se o que será encontrado é realmente novo, então é por definição algo desconhecido no princípio. Não há nenhum meio de dizer aonde levará um dado domínio de pesquisa. É por isso que não se podem escolher alguns aspectos da ciência e rejeitar os outros. (...) a ciência é algo que possuímos ou não possuímos. E se a possuímos, não podemos tirar dela somente aquilo de que gostamos. É necessário aceitar também o imprevisto e o perturbador. É inútil, portanto, ter a esperança de prever a direção que pode tomar uma ciência. A qualquer instante se pode, em função do conhecimento adquirido, imaginar o que vai acontecer nos (...) próximos cinco anos. Mas essa é a parte menos interessante da pesquisa: o dia-a-dia, a rotina. A parte verdadeiramente interessante é a que não se pode prever.”

François Jacob, 1998.

Sumário

Lista de abreviaturas

9-LOX: 9-lipoxigenase
ACC sintase: 1-aminociclopropano-1-carboxilato sintase
BA: ácido benzoico
C15d: Controle após 15 dias
C5d: Controle após 5 dias
CEPA: ácido 2-cloroetilfosfônico (Ethrel)
CYP450: complexo citocromo P450 monoxigenase
CYP736B: citocromo P450 736B
DEGs: Differentially expressed genes - genes diferencialmente expressos
DMAPP: dimetilalildifosfato
DTS: diterpeno sintase
ERF112: fator de transcrição de resposta a etileno
EROs: Espécies reativas de oxigênio
ET: ethrel
Et15d: Pasta adjuvante após 15 dias
Et5d: Pasta adjuvante após 5 dias
FC: Fold Change
GC/MS: cromatografia gasosa acoplada a espectometria de massas
GGDS: geranilgeranil difosfato sintase
HY: indivíduos de alta produção de resina
IPP: isopentenil difosfato
Iso: isoleucina
K: sulfato de potássio
LA: ácido linolênico
LY: indivíduos de baixa produção de resina
MeJa + Iso: metil jasmonato + isoleucina
MeJa: metil jasmonato
MEP: 2C-metil-D-eritritol-4-fosfato
MVA: mevalonato
Pc1TPS: (+)-α-pineno sintase
Pc2TPS: (−)-β- pineno sintase
Pc3a: abietadieno sintase
Pt3b: α-farneseno sintase
RNAseq: sequenciamento de RNA mensageiro (cDNA)
RT-qPCR: PCR quantitativa precedida por transcriptase reversa
SA: ácido salicílico
TAT: taxadienol acetil transferase
TPS: terpeno sintases

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RESUMO

O cultivo florestal de *Pinus elliottii* (Engelm.) é uma fonte sustentável de celulose, madeira e resina. A resina é uma mistura terpênica complexa e viscosa, que é secretada pelo tronco, cones e folhas em resposta a dano mecânico, atuando como barreira física no selamento do ferimento e, por vezes, no aprisionamento de insetos herbívoros. A resina é composta por moléculas voláteis e não voláteis capazes de inibir o crescimento de microrganismos, bem como repelir insetos e atrair agentes de relação tritrófica (predadores de herbívoros). Devido às propriedades físico-químicas deste material, a resina é um dos produtos não-madeireiros mais valiosos da indústria florestal, com aplicações em diversos segmentos, desde indústria farmacêutica, de cosméticos e alimentos, até produção de tintas, solventes, borrachas, adesivos, biocombustíveis, etc, capaz de substituir diversos produtos derivados do petróleo. A resina é extraída de árvores adultas com cortes periódicos transversais que removem a casca do tronco, expondo o câmbio e lenho recente. Sobre o dano é aplicada uma pasta indutora da biossíntese de terpenos, visando aumento de produção. Diversos estudos tratam da prospecção e validação de novas substâncias adjuvantes com capacidade indutora de resinose, almejando, principalmente, compostos de menor custo e toxicidade, mas com eficácia de indução de resina similar ou superior aos já utilizados comercialmente. No entanto, estes estudos são laboriosos, caros e demorados em função da condução de ensaios em condições de campo com elevado número de árvores adultas. Frente a este quadro, utilizamos plantas jovens com idades entre 1 e 3 anos como modelo experimental para possível identificação de novos adjuvantes indutores, em um sistema que denominamos “microrresinagem”. Plantas jovens responderam à indução de adjuvantes conhecidos como muito potentes em árvores adultas, embora as plantas de menor idade não tenham acusado diferença estatística em relação a indutores com potencial mediano de estimulação de resinose, mas ainda assim efetivos em indivíduos maduros. Ainda neste sistema experimental, foi possível identificar de modo precoce indivíduos com diferentes capacidades de produção de resina (altamente resinosos e pouco resinosos). Além disso, a análise dos perfis de expressão de genes de duas terpeno-sintases (α e β-*PINENO SINTASES*) e de um fator de transcrição responsivo a etileno (*ERF112*) mostrou que os mesmos têm potencial para diagnóstico molecular de fenótipo resinífero. Por outro lado, as bases moleculares de indução de resina por pastas indutoras comerciais à base de ácido e precursores sintéticos de etileno em plantas adultas de *P. elliottii* ainda são desconhecidas. Visando aumentar o entendimento dos processos moleculares subjacentes à resinose em *P. elliottii*, foi realizado um estudo transcriptômico comparativo entre plantas adultas controle (somente ferimento) e tratadas com pasta comercial (ferimento seguido de aplicação de pasta), 5 ou 15 dias após a realização da estriagem. Dentre as alterações observadas, destacaram-se resposta a dano oxidativo, restauração de parede celular e metabolismo de fitormônios, além de diversas respostas de defesa, revelando uma intrincada rede de regulação da produção de resina. O conjunto de resultados desta Tese lança luz sobre novos aspectos da resinagem comercial de *P. elliottii*, oferecendo ferramentas para o melhoramento genético e manejo florestal voltado à indústria de bioresina.

Palavras chave: resina, terpeno, pinus, transcriptoma, resinagem

ABSTRACT

Plantations of slash pine (*Pinus elliotii* Engelm.) are a sustainable source of cellulose, paper and resin. Resin is a complex and viscous blend of terpenes secreted by the trunk, cones and leaves as a result of mechanic damage, acting as a physical barrier sealing the wound and sometimes entrapping insect herbivores. Resin is composed of volatile and non-volatile molecules, which are capable of inhibiting the growth of microorganisms, as well as repel insects and attract agents of tritrophic relations (predators of herbivores). Due to its physico-chemical properties, resin is one of the most valuable non-wood products of the forestry industry, finding applications in several sectors, including pharmaceutical, cosmetic, and food industry, production of paints, solvents, rubber, adhesives, biofuels, among others, being able to replace various petroleum-derived products. Resin is extracted from adult plants periodically wounded with a transversal cut for bark removal and exposure of the cambium and early sapwood. A terpene biosynthesis stimulant paste is applied on the wound line to increase resin yields. Several studies deal with the screening and validation of new adjuvant substances with resinosis stimulation capacity, targeting low cost and reduced toxicity compounds, but that are still able to stimulate resin exudation at equivalent or superior levels compared to those currently commercially used. Nonetheless, these studies are rather laborious, expensive and time-consuming, as a function of the need to carry out assays under field conditions with a high number of adult trees. With this scenario in mind, we used young trees with ages ranging from 1 to 3 years as an experimental model to identify new paste adjuvants, in a system we called 'microtapping'.Young trees responded to resin induction by powerful adjuvants, although they did not show statistical differences in resin yield with inducers know to be of medium potency, but successfully used in field adult trees for resinosis stimulation. This system also allowed the precocious identification of individuals with different capacities of resin production (highly and poorly resinous). Moreover, the analyses of gene expression profiles of two terpene synthases (*α* and *β-PINENE SYNTHASES*) and an ethylene responsive transcription factor (*ERF 112*) showed that these have diagnostic potential for the resiniferous phenotype. On the other hand, the molecular bases of resin induction by commercial stimulant pastes using acid and a synthetic precursor of ethylene in adult slash pine trees are still largely unknown. With the purpose of expanding the understanding of the underlying processes involved in slash pine resinosis, a transcriptomic study was conducted comparing control (wound only) and commercial paste-treated adult trees (wound plus paste application), 5 and 15 days after bark streaking. Among the observed changes, a few were highlighted, such as oxidative damage response, cell wall restoration, and phytohormone metabolism, as well as several defense responses, uncovering an intricate network of regulation of resin production. The combined findings of this thesis shed light on novel aspects of commercial resin tapping in slash pine, supplying potential tools for genetic improvement and forest stand management aiming at bioresin supply.

Key words: resin, terpene, pinus, transcriptome, tapping

Bases Moleculares da Resinagem em *Pinus elliottii* Engelm

I. Introdução

Ao passo que metabólitos primários são essenciais ao funcionamento basal das células e ubíquos, metabólitos secundários (oriundos do metabolismo primário e também conhecidos por produtos naturais e, mais recentemente, metabólitos especializados) são necessários nas respostas dos organismos ao ambiente, possuindo papel proeminente nas interações ecológicas e na adaptação às condições externas (Matsuura et al., 2018). O metabolismo especializado é bastante evidente em organismos sésseis, como fungos, poríferos, celenterados e plantas, os quais necessitam responder *in situ* de modo adaptativo aos estresses e outros sinais e alterações do ambiente. Dentro de cada grupo de organismos, a distribuição de diferentes compostos secundários e mesmo vias metabólicas inteiras é geralmente restrita a alguns taxa, o que tem sido usado em estudos evolutivos e de sistemática no ramo da quimiotaxonomia.

Em plantas, além de envolvidos na adaptação a estresses bióticos (*e.g.* defesa contra herbívoros e patógenos) e abióticos (*e.g.* seca, alagamento, salinidade, extremos de temperatura e irradiância, desequilíbrios em nutrientes minerais e presença de metais pesados ou outros poluentes), metabólitos especializados também servem para atrair dispersores de sementes, polinizadores ou organismos simbiontes (como micorrizas e bactérias fixadoras de nitrogênio), que auxiliarão na adequação ao meio externo (Abbas et al., 2017; Pichersky and Lewinsohn, 2011). Metabólitos secundários compreendem diversas classes químicas, como alcaloides, antocianinas, glicosinolatos, terpenos, fenólicos, entre outros (Mithöfer and Boland, 2012), e exigem alto gasto

energético ao organismo que os produz (Züst and Agrawal, 2017). Desta forma, a concentração de metabólitos resultantes destas vias geralmente é baixa em condições normais. No entanto, a síntese destes compostos sofre complexa regulação gênica, podendo muitas vezes ser fortemente induzida sob condições específicas, tanto para estresses bióticos quanto abióticos (Prinsloo and Nogemane, 2018; Yang et al., 2018). Fitormônios de defesa, como ácido salicílico (Boatwright and Pajerowska-Mukhtar, 2013) e jasmonato (Wasternack and Strnadb, 2019), além de etileno (Khan et al., 2017; Schaller, 2012) são relacionados à indução de síntese de diversos metabólitos.

Os metabólitos secundários de plantas são amplamente conhecidos pelo seu potencial na saúde humana, havendo diversos relatos históricos sobre sua relevância na medicina popular em diversas culturas (Afrin et al., 2015). A despeito de avanços expressivos em síntese química, quase metade das drogas que foram aprovadas para uso medicinal entre 1981 e 2014 são produtos naturais ou produtos deles derivados ou neles inspirados (Newman and Cragg, 2016). A biodiversidade de plantas representa uma reserva incomensurável de compostos com potencial ainda a serem explorados e identificados (Guerriero et al., 2018). Muitas vezes estas moléculas são de alta complexidade estrutural, além de serem caras ou dificilmente obtidas por síntese química. Em função disso, e porque plantas são organismos relativamente fáceis de manipular, diversos estudos são realizados na tentativa de compreender a fisiologia por trás destes processos biológicos, bem como estabelecer métodos de potencializar sua produção (Matsuura et al., 2018).

Terpenos compõem a maior classe de metabólitos secundários encontrados na natureza, com mais de 40 mil compostos já identificados (Chen

et al., 2011). A biossíntese destas moléculas em plantas é essencial para o seu desenvolvimento e envolve duas vias metabólicas: a via do mevalonato (MVA), que ocorre no citosol, e a via do 2C-metil-D-eritritol-4-fosfato (MEP), que ocorre em plastídios (Vranová et al., 2013). Em ambas são produzidas moléculas de isopentenil difosfato (IPP) e di-metil-alil difosfato (DMAPP) (Figura 1). A isomerização de uma molécula de DMAPP e um número variável de moléculas de IPP leva à geração de compostos tanto do metabolismo primário quanto secundário, como geranil difosfato e monoterpenos (C10), farnesil difosfato e sesquiterpenos (C15), geranil-geranil difosfato e diterpenos (C20), além de diversos outros compostos como ácido abscísico, giberelinas, citocininas, strigolactonas, brassinosteroides, tocoferóis, plastoquinonas, entre muitos outros (Pichersky and Raguso, 2018). Terpenos resultantes da ação de terpeno sintases, envolvidas na ciclização de cadeias carbonadas, sofrem alterações estruturais, como oxidações, reduções, conjugações e substituições, geralmente realizadas por enzimas do complexo citocromo P450 monoxigenase, as CYP450 (Mizutani and Ohta, 2010; Shalan et al., 2018). Essas modificações geram uma ampla variedade de moléculas com propriedades químicas distintas e são responsáveis, em parte, pelo grande número de compostos terpênicos produzidos pelas plantas (Schuler, 1996; Weitzel and Simonsen, 2013).

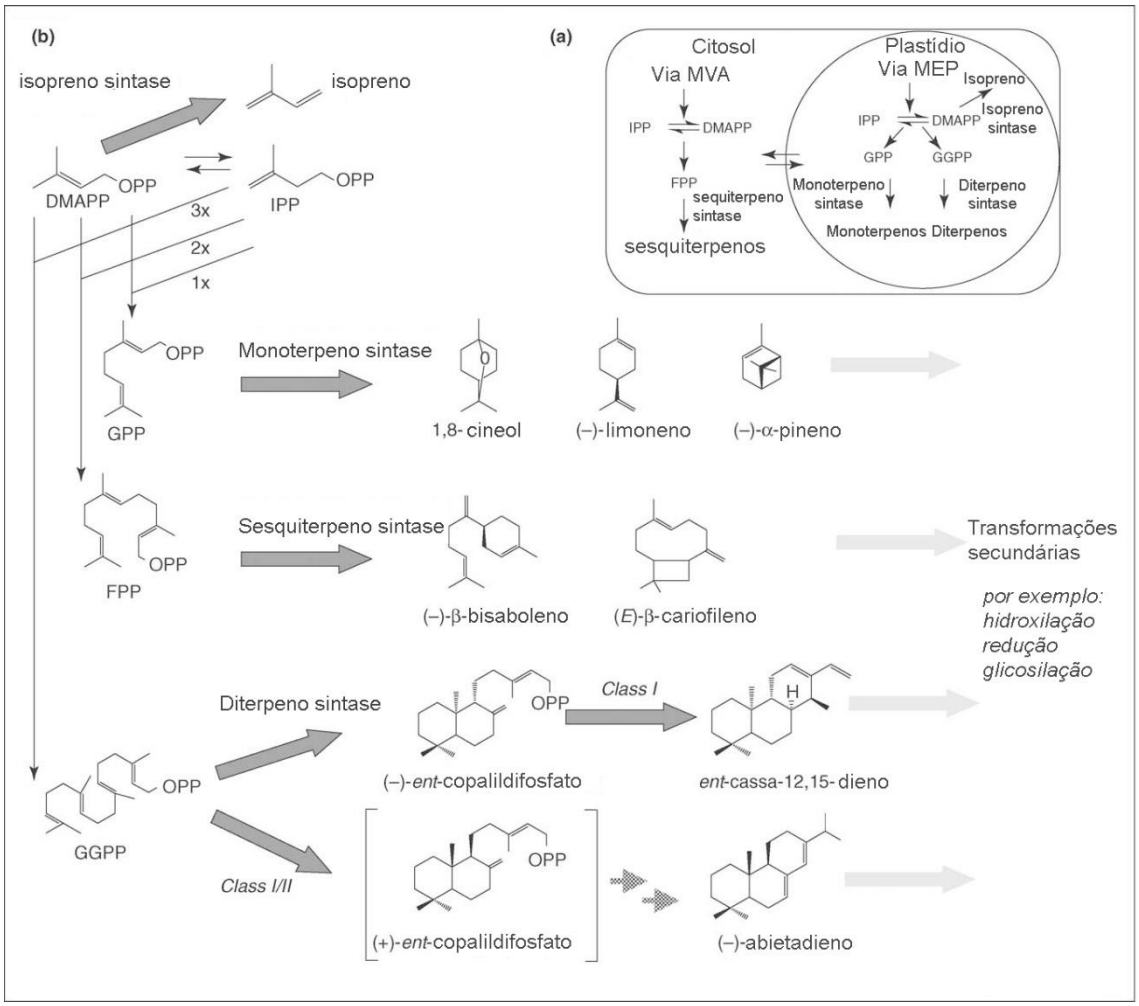

Figura 1.a. Compartimentação subcelular das vias MVA e MEP em células vegetais. Unidades de C5 de DMAPP e IPP são formadas por duas vias metabólicas independentes, a via do mevalonato (MVA) e do metileritritol fosfato (MEP), que ocorrem no citosol e plastídio, respectivamente. **b.** A isomerização de uma molécula de DMAPP e número variável de IPP, dá origem aos precursores terpênicos. Todos os terpenos são derivados de geranil diosfato (GPP), farnesil difosfato (FPP) e geranilgeranil difosfato (GGPP) pela atividade das terpeno sintases (TPS). OPP indica a porção difosfato (adaptado de: Tholl, 2006).

Coníferas estão entre as espécies arbóreas mais antigas a dominar diversos ambientes terrestres, e muitas árvores chegam a idades centenárias (Pascual et al., 2015). Durante os milhares de anos de evolução, estas plantas desenvolveram um sistema de defesa contra estresses ambientais bastante intrincado e dinâmico, baseado em barreiras químicas e físicas (Pascual et al., 2016; Trapp and Croteau, 2001). Quando atacadas, os ferimentos provocados nas cascas resultam na secreção de resina, uma mistura complexa de terpenos (Philipps and Croteau, 1999). Este material é composto por uma fração volátil, formada por monoterpenos e sesquiterpenos, que serve para deterrência de herbívoros, atratores de predadores de herbívoros (interação tritrófica), além de possuir atividade antimicrobiana (Lange, 2015) e uma fração sólida, denominada rosina, composta por diterpenos, que oxida em contato com o ar e resulta no selamento do dano e eventual aprisionamento de insetos (Keeling and Bohlmann, 2006). Devido a este mecanismo de resposta que atua em diversos níveis de defesa, a produção de resina, além de ser em parte constitutiva, é altamente induzida por estresses bióticos, abióticos e sazonais (Ferrenberg et al., 2014; Franceschi et al., 2005; Hall et al., 2013; Lai et al., 2017; Rodrigues-Corrêa and Fett-Neto, 2012; Rodrigues-Corrêa and Fett-Neto, 2013; Rodrigues and Fett-Neto, 2009; Rodríguez-García et al., 2014; Rodríguez-García et al., 2015; Schiebe et al., 2012; Seybold et al., 2006; Westbrook et al., 2013).

Em coníferas, a resina constitutiva é mantida em estruturas anatômicas especializadas em formato de tubos, denominadas dutos resiníferos, presentes nos tecidos vasculares de caules e folhas (Lange, 2015). Essas estruturas servem como reservatórios pressurizados de armazenamento, que extravasam seu conteúdo quando rompidas por dano mecânico, como ataque de herbívoros

(Zulak and Bohlmann, 2010). Revestindo estes dutos, encontram-se as células responsáveis pela biossíntese e secreção de resina, ricas em leucoplastos não fotossintetizantes associados ao retículo endoplasmático (Lange, 2015). Transportadores do tipo ABC atuam no carregamento dos dutos resiníferos com terpenos (Liu et al., 2015). Assim como a resina, a produção de novos dutos resiníferos pode ser induzida por ataque de insetos, ferimentos, exposição a patógenos ou tratamento com mimetizadores de estresse biótico, como metil-jasmonato (Ferrenberg et al., 2014; Hudgins et al., 2004; Martin et al., 2002; McKay, 2003; Nagy et al., 2000; Rodríguez-García et al., 2016).

Há muito tempo a humanidade faz uso dos produtos obtidos da resina de coníferas. Desde tempos ancestrais, a resina tem sido utilizada para a produção de adornos, calafetagem de navios e impermeabilização de madeira (Bohlmann and Keeling, 2008). Atualmente, os derivados de resina encontram aplicação em indústria de tintas, adesivos, inseticidas, solventes, fragrâncias e cosméticos, flavorizantes alimentícios, além de servirem de precursores de moléculas ativas usadas na indústria farmacêutica e química fina (Rodrigues et al., 2013; Yadav et al., 2015). Todos estes usos tornam a resina um dos produtos não-madeireiros mais valorizados da indústria florestal (Neis et al., 2019).

O Brasil é um dos principais produtores de resina no mundo, com uma produção de 185.692 toneladas em 2018 (ARESB, 2019). As florestas para tal finalidade se concentram principalmente nos estados de São Paulo, Rio Grande do Sul, Paraná e Minas Gerais. Embora aproximadamente 84% da produção seja oriunda de plantações de *Pinus elliottii* var. *elliottii*, *Pinus caribaea* var. *hondurensis* também é explorada pela indústria florestal no país. Toda a produção de resina no Rio Grande do Sul provém de plantações de *P. elliottii*,

que conta com florestas plantadas e/ou regeneradas do banco de sementes (AGEFLOR, 2019). O estado foi responsável pela produção de 45.720 toneladas de resina em 2018, correspondendo a 24,6% da produção nacional (ARESB, 2019). Até o momento, não há programas de melhoramento genético para esta espécie visando produção de resina, e as florestas comerciais não são clonais. De fato, a diversidade genética encontrada entre as árvores é bastante grande, ocasionando uma ampla diferença na produção de resina por árvore, que pode variar de 2 a 8 kg de resina anualmente (Neis et al., 2018).

Pinus elliottii é uma espécie oriunda do Sul e Sudeste dos Estados Unidos e se adaptou bem à região Sul e Sudeste do Brasil. Sua madeira não é da melhor qualidade para a indústria de papel e celulose, mas apresenta exsudação abundante de resina em cortes e ferimentos na madeira, ramos e acículas (Yadav et al., 2015). Seus cones são pedunculados, com escamas e sem espinhos. Quando adultas, atingem de 18 a 30 m de altura e circunferência do caule entre 65 e 100 cm (Little and Dorman, 1952). Apesar de seus diversos usos, esta conífera é também reconhecida por seu potencial invasor, ligado a resiliência de suas formas jovens e à ampla dispersão de suas sementes por anemocoria. Em vista disso, no estado do Rio Grande do Sul, o código florestal estadual não permite o estabelecimento de pinus em novas áreas de terra, ficando as florestas plantadas restritas às suas atuais áreas de plantio. Por conseguinte, há uma clara necessidade de aumentar a produção de resina em plantações de florestas já existentes no estado.

Comercialmente, a resina é obtida de florestas de árvores adultas, geralmente a partir de 14 anos de idade e se mantém até o momento do corte da árvore (Ferreira et al., 2011). Um corte transversal é realizado a cada duas

semanas na casca das árvores e a resina exsudada é coletada periodicamente (Rodrigues et al., 2008; Rodríguez-García et al., 2016). Sobre o ferimento é aplicado uma solução contendo compostos que resultam no prolongamento do dano ou induzem a síntese de terpenos de resina, denominada pasta adjuvante ou pasta indutora (Figura 2) (Fuller et al., 2016). A composição desta pasta varia para cada empresa florestal, mas em geral é composta por um agente causador de dano (usualmente ácido sulfúrico 20% v/v) acrescido de um precursor sintético do hormônio etileno (ácido 2-cloroetilfosfônico, CEPA, também conhecido como Ethrel, 3% v/v) (McReynolds and Kossuth, 1984). Devido ao alto custo e limitações regulatórias deste composto, no entanto, esforços vêm sendo feitos na tentativa de prospectar novos adjuvantes (Rodrigues-Corrêa et al., 2012). Diversos estudos já demonstraram que compostos como cofatores enzimáticos de terpeno sintases ou de receptores de etileno (cobre, ferro, magnésio, manganês e potássio), extrato de levedura, hormônios vegetais (como ácido salicílico, jasmonato ou auxinas) ou seus precursores (ácido benzoico, nitroprussiato de sódio) apresentam efeito indutor de resinose em algumas espécies comercialmente exploradas (Figura 3) (Neis et al., 2018; Rodrigues et al., 2011; Rodrigues and Fett-Neto, 2009).

Embora apresentando resultados importantes, trabalhos envolvendo plantas de *P. elliotti* a campo e prospecção de novos estimulantes de resinagem têm se mostrado laboriosos e demorados, envolvendo de centenas a milhares de árvores por períodos de alguns anos de avaliação, além de extenso trabalho de campo (Lai et al., 2017; Neis et al., 2018; Rodrigues et al., 2011; Rodrigues and Fett-Neto, 2009). A busca por alternativas para programas de melhoramento da espécie e seleção de novos adjuvantes em menor tempo e sob condições

controladas passa pela validação de suas respostas em plantas jovens. Além disso, faz-se necessário o entendimento das bases moleculares envolvidas na indução de resinagem em plantas adultas, que ainda é desconhecida em espécies florestais de valor comercial à indústria resinífera como *Pinus elliottii*, o que seria de grande valia para programas de melhoramento e manejo de plantações florestais visando maior produtividade por árvore.

Figura 2. Representação esquemática estriagem de pinheiros para produção de resina. Um ferimento é realizado para remoção da casca e exposição do câmbio vascular. Em seguida, uma pasta estimulante é aplicada no dano. A resina exsudada é coletada em sacos plásticos (adaptado de: de Lima *et al*., 2016).

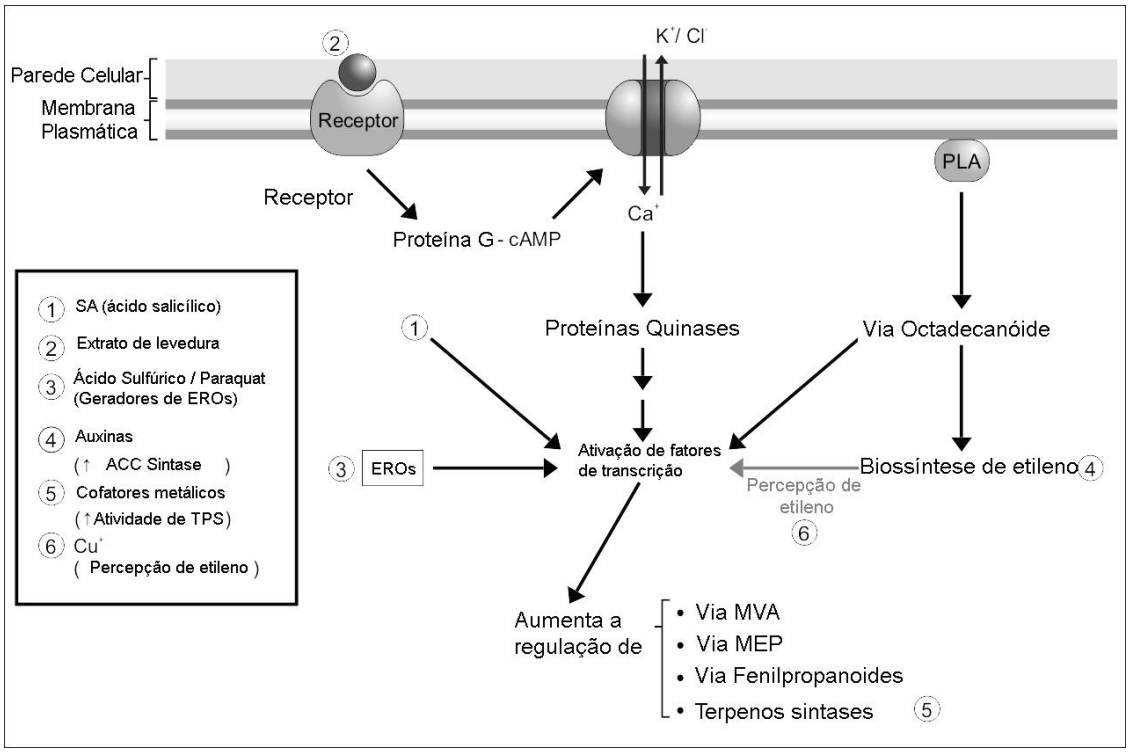

Figura 3. Possível mecanismo de defesa induzido em coníferas, cuja sinalização é explorada pela resinagem comercial para aumentar a produção de compostos de resina (adaptado de: Rodrigues-Corrêa e Fett-Neto, 2012).

II. Hipóteses

a. A resinagem de plantas jovens de *Pinus elliottii* de 1 até 3 anos de idade (microrresinagem) pode ser usada para testar potenciais adjuvantes indutores de resinose, bem como para identificar indivíduos de maior potencial resinífero.

b. O estriamento do tronco (remoção da casca e exposição do lenho recente) e a aplicação de pasta estimulante de resina acionam um programa de expressão gênica associado a ferimento, estresse oxidativo e síntese de terpenos, que conjuntamente culminam na resinose localizada em árvores adultas de *Pinus elliottii* cultivadas em condições de campo.

III. Objetivos

1. Estabelecer e validar um método de resinagem de *Pinus elliottii* jovens com potencial para identificação de novas pastas estimuladoras de resinagem e seleção indivíduos de alto potencial resinífero de modo mais rápido, barato e menos laborioso do que usando indivíduos adultos em campo.

2. Caracterizar e analisar o transcriptoma de árvores adultas de *Pinus elliottii* durante o processo de resinose induzida por procedimentos convencionais de resinagem em condições de campo, visando aperfeiçoar estratégias de melhoramento genético e de manejo de plantações.

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IV. 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** contém o manuscrito submetido ao periódico *Industrial Crops and Products* (IF: 3.849). Este capítulo trata sobre a utilização de plantas jovens de *Pinus elliottii* (com idades entre 1 e 3 anos), cultivadas em ambiente controlado, como modelo na seleção de novos indutores de resinagem em plantas adultas. Além disso, apresentamos uma metodologia baseada em microresinagem e expressão gênica por PCR em Tempo Real que pode auxiliar na identificação precoce de indivíduos com perfil contrastante na produção de resina.

- O **Capítulo 2** contém o manuscrito submetido ao periódico *Industrial Crops and Products* (IF: 3.849). Este capítulo trata do perfil de genes diferencialmente expressos durante o processo de resinagem comercial de árvores adultas de Pinus elliottii. Comparamos os efeitos produzidos pela utilização de adjuvante utilizado pela indústria florestal (contendo precursor sintético de etileno) e pelo tempo transcorrido após a realização dos danos. Os resultados foram obtidos por sequenciamento de RNA, expressão relativa por PCR em Tempo Real e quantificação dos terpenos majoritários de resina por cromatografia gasosa acoplada a espectrômetro de massas.

- O **Anexo 1** contém o resumo do artigo publicado no periódico *Industrial Crops and Products* (IF: 3.849) que trata da seleção de indivíduos adultos de *Pinus elliottii* com perfis contrastantes de produção de resina baseado em metodologias de vazão de resina, anatomia de dutos resiníferos e quantificação de terpenos majoritários de resina por cromatografia gasosa acoplada a espectrômetro de massas.

- O **Anexo 2** contém o resumo do artigo publicado fora do escopo da tese no periódico *Frontiers in Plant Science* (IF: 3.677), e trata das bases bioquímicas, fisiológicas e moleculares relacionados à perda da capacidade de indução de enraizamento adventício em explantes de *Eucalyptus globulus* cultivados *in vitro*.

- O **Anexo 3** contém o resumo do artigo publicado fora do escopo da tese no periódico *Environmental and Experimental Botany* (IF: 3.666), e trata da indução do alcaloide braquicerina em Psychotria brachyceras por temperatura e seu efeito mitigador de estresse oxidativo, tanto *in situ* quando sobre aplicação em outras espécies vegetais.

- O **Anexo 4** contém o resumo do Capítulo de Livro “Environmental regulation of bioactive metabolite accumulation in Brazilian medicinal plants”, a ser publicado fora do escopo da tese pela editora CRC Press no livro “*Brazilian Medicinal Plants*” em um volume especial da série “Natural Products Chemistry of Global Plants”, editado pelo Dr. Raymond Cooper.

- Os **Anexos 5** e **6** contém os certificados de premiação que foram obtidos pelos trabalhos presentes nesta tese, respectivamente, pelo aluno de iniciação científica João Vitor Vigne Duz (menção honrosa na sessão de pôsteres no Sul Biotec 2018 com resultados parciais do Capítulo 1) e pela autora da tese (destaque de apresentação oral no Sul Biotec 2018 com resultados parciais do Capítulo 2).

V. Capítulo 1.

Resinosis of young slash pine (*Pinus elliottii* Engelm.) as a tool for resin stimulant paste development and high yield individual selection

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Highlights:

- Resin tapping of young pine (microtapping) helps prospection of new stimulants

- Paste adjuvants with high resin stimulatory potential have effect on young plants

- Young pines respond to periodic stimuli for resin production akin adult trees

- Microtapping and gene expression help early selection of high resin yield pines

Abstract

Pine resin, a natural source of industrially relevant terpenes, is a major non-wood forestry commodity. Resin is obtained by wounding the bark of adult trees and applying stimulant pastes with different adjuvants on the wound. Identifying new adjuvants and high resin producing trees in adult forests often requires long time and intense labor. Microtapping, *i.e.* use of young plants of *Pinus elliottii* var. *elliottii* cultivated in greenhouse to extract resin, was evaluated as an alternative to carry out these activities. Compounds with known effect in adult plants (ethrel, benzoic acid and potassium sulfate) and molecules involved in the transduction of defense signals (methyl jasmonate, salicylic acid, linolenic acid and isoleucine) were evaluated in young plants. One, two and three-year-old plants consistently increased resinosis when treated with potent adjuvants, mainly methyl jasmonate. The more lignified basal stems produced more resin than apical ones in the 1-year-old plants. Resin yield increased after the second year. All plants were responsive to successive stimuli, just as adult plants. High resin-yield individuals were identified by microtapping, and this phenotype was further supported by terpene-related gene expression studies associated with resinosis. Therefore, microtapping can be used for early identification of adjuvants with high resin induction capacity and of putative elite individuals for field evaluation, breeding, and clonal propagation.

Keywords: resinosis, adjuvant paste, *Pinus elliottii*, young plants

1. Introduction

Conifers are the most advanced group of gymnosperms that also include some of the longest living species on the planet, with individual trees often exceeding several hundred years (Warren et al., 2015). The adaptive success and co-existence with changing environmental conditions, competing plants, potential pests and foraging animals was only possible due to the acquisition of anatomical and chemical defense systems. This systems include sophisticated constitutive and inducible mechanisms, that involve structural, morphological or physical barriers in all major organs and different tissues (Pascual et al., 2015; Pham et al., 2014; Warren et al., 2015). Resin is a viscous fluid exuded from ducts when the tree is under herbivore or pathogen attack (Lange, 2015). The constitutive and induced resin are considered the major chemical defense of conifers, and their composition is a complex, dynamic and variable mixture of terpenoids such as monoterpenes, sesquiterpenes, and diterpenes (Bohlmann and Keeling, 2008; Franceschi et al., 2005; Philipps and Croteau, 1999; Zulak and Bohlmann, 2010).

Pine resin is the raw material for several industrial products and one of the most important non-timber forest products (Neis et al., 2019). The total annual production reaches about 1.2 million tons worldwide, supporting a wide range of multi-billion-dollar industrial applications (Yadav et al., 2015). Because it is an easy to obtain, inexpensive and renewable source material, pine resin and its products are used in the production of fungicides, insecticides, fragrances, paints and solvents, adhesives, rubber, biofuels, and especially in fine chemicals such as biodegradable polymers, precursors of drug synthesis and food additives (Neis et al., 2019; Yadav et al., 2015).

Brazil is one of the world leaders in pine resin production, mostly based on slash pine (*Pinus elliottii* var. *elliottii*) plantations of the Southeast and South regions (ARESB, 2018). Under normal growing conditions, pines accumulate between 1 and 5% of their stem mass as resin, but after treatment with chemical elicitors of resinosis the stem oleoresin content generally increases significantly (Rodrigues-Corrêa and Fett-Neto, 2012; Westbrook et al., 2013). The exudate resin is collected from a transverse wound mechanically imposed to the bark of the adult tree (bark stripping), followed by application of adjuvant paste on the damage upper line, to promote the biosynthesis and flow of terpenes (Fuller et al., 2016; Rodrigues et al., 2013).

Commercial adjuvant pastes are, in general, made of a combination of sulfuric acid (20% v/v) (to prolong wounding and increase oxidative stress) and 3 to 4.5% of Ethrel (also known as CEPA, 2-chloroethylphosphonic acid), an ethylene precursor that stimulates resin production and flow (McReynolds and Kossuth, 1984; Rodrigues-Corrêa and Fett-Neto, 2012), as well as a carrier material to provide adhesion on the wound (*e.g.* rice husks and silica) (Fuller et al., 2016). In order to improve resin production and reduce overall costs, there is an interest in prospecting new adjuvant compounds, which are often defense-inducing molecules (Rodrigues-Corrêa et al., 2012; Rodrigues et al., 2011; Rodrigues and Fett-Neto, 2009).

Adjuvants may include phytohormones, such as ethylene, auxin, salicylic acid, and jasmonate, precursors of some of these molecules (benzoic acid, ethrel, ethephon), or metal cofactors of terpene synthases and ethylene receptors (iron, potassium, magnesium, manganese, copper) (Fuller et al., 2016; Neis et al., 2018; Perotti et al., 2015; Rodrigues-Corrêa and Fett-Neto,

2013; Rodrigues et al., 2011). However, due to genetic and environmental factors, fieldwork for the validation of new compositions is expensive, laborious and time-consuming (Neis et al., 2018). The present work aimed to evaluate the potential of tapping young plants of slash pine (microtapping) for selecting new adjuvants that could be used for resin production and precocious selection of putative high yield individuals, thereby reducing time and costs. Moreover, we examined the expression profile of select terpene biosynthesis-related genes in low and high resin yield individuals selected by microtapping as a validation and additional tool for the early selection of elite individuals.

2. Material and Methods

2.1 Plant material and growth conditions

Six-month-old plants of *P. elliottii* were purchased from a local nursery. Seedlings were transferred to plastic cups with 700 mL capacity with commercial organic soil and vermiculite (1:1) for six months in a growth room (23 ºC ± 2, photoperiod of 16 h, irradiance of 80 µmol.m^{-2}.s^{-1} supplied by fluorescent lamps). Plants were regularly watered as needed and once a month received 0.1x of macro and micronutrients solution of MS (Murashige and Skoog, 1962). At the beginning of the experiment, one-year-old plants (herein referred to as seedlings) had approximately 0.5 cm of diameter at basal stem height. Three months after the end of the first experiment the plants were transferred to greenhouse and conditioned in PVC pots containing the same substrate composition as described above. Plants were periodically watered as needed until reaching 2 and 3 years of age (herein called saplings) with diameter at basal stem height of approximately 1.6 cm) for the other set of tests. Plants were protected from excess sunlight with a black shade net and

irradiance was provided by natural sunlight (at plant level ranging from about 500 to 900 μmol.m^{-2}.s^{-1} on a typical day). Day length was approximately 12 h and temperature ranged from 14 to 26 ºC during all of the experiments.

2.2 Experimental sequence and chemical stimulant treatments

The experiments were carried out in 3 phases: the first, with one-year-old plants in a growth room, the second and third with the same plants, at age two- or three-years, under greenhouse conditions. With the aid of a scalpel, wounds of approximately 1 cm were made to expose the cambium (micropanel method) in the basal, median and apical portions of the stem, starting from the first brachyblast or branch insertion at the base, and then moving up counterclockwise towards the apex, avoiding trunk annealing. Initially, preliminary experiments were carried out with the micropanel tapping method with different concentrations of glycerol (10, 25 or 50% v/v) with methyl jasmonate (MeJa) at 100 mM as adjuvant and also with or without sulfuric acid 5% (v/v). For the first paste tests, 50 μL of one of the adjuvants was applied on each wound. The adjuvants consisted of aqueous and glycerol solution 1:1 (v/v) added of benzoic acid (BA) at 50 mM (a precursor of salicylic acid, SA), ethrel (ET) at 100 mM (releaser of ethylene and stimulator of resin biosynthesis and exudation), potassium sulfate (K) at 500 mM (activator of gymnosperm terpene synthases) or (MeJa) at 100 mM (stimulator of resin biosynthesis). As negative control, only the water-glycerol vehicle was applied. Resin released at each point was collected weekly for six weeks and weighed. The pastes were reapplied in the third week of experiment, without any additional injury being inflicted. For each treatment, 36 or 37 plants were used, totaling 185 individuals.

One year after the end of the first experiment, 72 plants that presented resin production within the standard deviation for each treatment were randomly mixed and then used in a second test with new pastes. Wounds were performed at three points as described previously, and each incision received 50 µL of aqueous and glycerol solution 1:1 (v/v) adjuvanted with one of the treatments: ET at 100 mM, linolenic acid (LA) at 200 mM (precursor of JA – jasmonate and MeJa), isoleucine (Iso) at 100 mM (forms active conjugate with jasmonate), MeJa at 100 mM, and combination of MeJa and Iso (MeJa + Iso - 100 mM + 100 mM). As negative control, only the water-glycerol vehicle was applied. Resin produced at each point was collected weekly for four weeks and weighed. The pastes were reapplied on the same wound after resin collection at second week. Twelve plants were used for each treatment.

The same plants used in the second experiment were randomly mixed again and subject to another round of tests at three years of age. Wounds were performed only at basal trunk, and each incision received 50 uL of aqueous and glycerol solution 1:1 (v/v) added of one of the following adjuvants: SA at 14.5 mM and MeJa at 10, 25, 50 or 100 mM. As negative control, only the water-glycerol vehicle was applied. In addition, five brachyblasts were removed at different portions of each plant and the same pastes used in the micropanel damage were applied at the points of brachyblast removal (brachyblast pick method). Resin produced (or resin drop exuded, for brachyblast picks) was collected weekly for four weeks and weighed. The pastes were reapplied on the same wound after the harvest during the second week. Seven plants were used for each treatment.

2.3 Gene expression analyzes

Based on previous micropanel tests during their first year, 3-year-old plants with extreme phenotypes of resin yield (low and high resin yield, LY and HY, respectively) were selected; 4 plants were included in each category and their resin yield was harvested and weighed along the gene expression studies for further confirmation of resinosis phenotype. For the gene expression experiments, a portion of the bark 5 cm above and below the wound (produced by the micropanel method) was removed and the exposed cambium and sapwood were collected for RNA extraction (1 week after 50 mM MeJa application in all of the trees of both resin phenotypes). Samples were immediately stored in liquid nitrogen after collection. Total RNA was extracted from the tissues using *PureLink RNA Kit* (Thermo Fisher), as described by de Lima et al. (2016b). Nanodrop spectrophotometer (Thermo Scientific) was used to quantify RNA concentration. Gel electrophoresis in 1% agarose was also performed to check RNA quality. Samples containing extracted RNA from each individual were stored at -80°C.

For cDNA synthesis, 500 ng of RNA from each sample were diluted in Milli-Q water, to a total volume of 8 µL. Sample treatment, incubation and equipment were as described by de Lima et al. (2016b). RT-qPCR analyses were performed using four biological replicates for each individual, in technical triplicates for each primer. The procedure was carried out using 48-well reaction plates with 0.1 ml (MicroAmp™ Applied Biosystems) containing 2 pairs of primers (de Lima et al., 2016a). Reactions were performed in a total volume of approximately 20 µL, as previously described (de Lima et al., 2016a). Data were analyzed by the comparative quantitative cycle method, and the PCR efficiency

from the exponential phase (Eff) was calculated for each individual amplification plot using the LinReg software (Ruijter et al., 2009). PCR average efficiency was determined for each amplicon. Reference genes *Histone 3* and *Ubiquitin* (de Lima et al., 2016a), target genes whose expression was analyzed, and corresponding primers used to amplify their transcripts are listed in Table 1.

Table 1. Description of target and reference genes, primers and expected amplicon size.

Name	*Gene*	Function	Primer sequence (5'-3') (forward/reverse)	Amplicon length (bp)
Pc1TPS	*(+)-α-pinene synthase*	(+) α-pinene synthesis	AGGTTGCCTACGGATGTCAG/ TGGTATCTTCTATGCTCCGAATC	101
Pc2TPS	*(−)-β-pinene synthase*	(-) β-pinene synthesis	GAGCTTCTCAAACCCGACAG/ GGAGGGTTCTCATCACCAAA	148
Pt3b	*α-farnesene synthase*	α-farnesene synthesis	TGGGAAGCTTTAATCGATGC/ GGAGAGTGGCTGCTCGATAC	124
Pc3a	*abietadiene synthase*	abietadiene synthesis	GAATGCTCTGGAGGATACGG/ TCCAGCCTTGGCATACTTCT	114
ERF112	*ethylene-responsive transcription factor ERF112*	Ethylene responsive transcription factor	TACAGAGGCGTAAGGCAGAG/C GACTTCCCCTGAATCTCAA	152
HISTO3	*histone 3*	Cell proliferation, DNA Binding, RNA methylation	GCTGAGGCTTACCTTGTG/ CCAGTTGTATATCCTT AGGCATAA	94
UBI	*ubiquitin*	Protein degradation, translation, DNA repair, endocytosis, protein traffic	GATTTATTTCATTGGCAGGC/ AGGATCATCAGGATTTGGGT	149

2.4 Statistical analyses

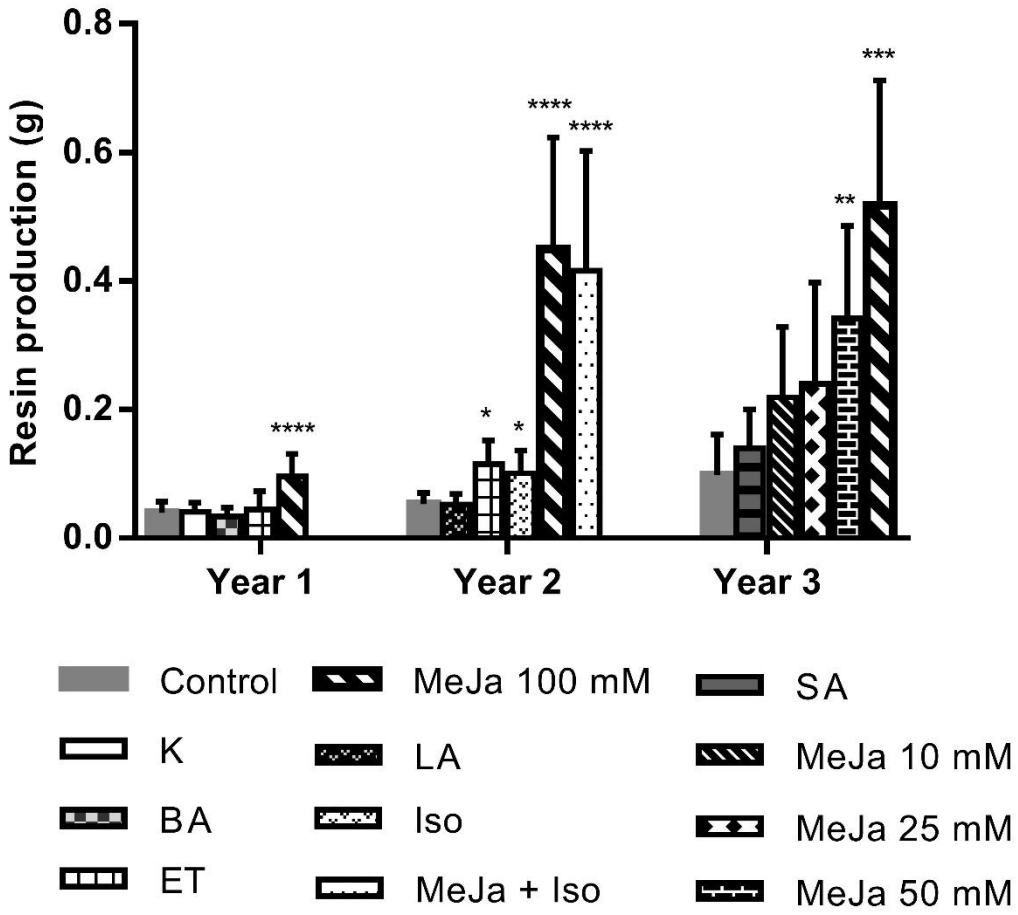
Experiments followed a totally randomized layout. Data were submitted to t-test against the control or ANOVA followed by Tukey tests when appropriate. For data sets without homogeneity of variance even after conventional data transformation protocols, nonparametric Welch's ANOVA was applied followed by Dunn's test. In every case, $p \leq 0.05$ was used. Tests were performed with R package version 1.3.5. and graphics were prepared using Graphpad Prisma software version 7.00.

3. Results

3.1 Resin yield and different stimulant pastes

Preliminary tests showed that sulfuric acid incorporation did not cause increased resin production when compared to the negative control (Figure S1). Concentration of glycerol in paste was also tested without significant difference in the resin yield (Figure S2). Subsequent tests indicated that incisions at points of brachyblast formation led to higher resin production than incision in stem portions devoid of them, which is why these spots at each stem portion were chosen to carry out injuries in all experiments. The total resin production at the end of the one-year-old plant experiments was not significantly different between the treatments in relation to the control, except for MeJa (Figure 1). However, 2-year-old saplings treated with ET, Iso and MeJa showed significant stimulation of resinosis, with no effect of LA (Figure 1).

In 3-year-old saplings, in which SA and low concentrations of MeJa were evaluated, only the higher concentrations of the MeJa were able to significantly increase resin yield over the control. It was also observed that plant age was associated with higher resin yield. Increase of 1.3 (control), 2.6 (ET) and 4.7-fold (MeJa) in the mean resin yield was observed when comparing similar treatments between years 1 and 2; 2.5 (control) and 5.4-fold (MeJa 100 mM) between years 1 and 3; and 1.85 (control) and 1.15-fold (MeJa 100 mM) between years 2 and 3. These age-dependent responses can be readily visualized for control and 100mM JA individuals (Figure S3).



188

189 **Figure 1.** Total resin exuded from 1-, 2-, or 3-year-old *P. elliottii* plants.
190 Data shown as means ± s.d. Dunn-test was applied against control in each set
191 of experiments (age of evaluation). *p < 0.05, **p < 0.01, ***p < 0.001, ****p <
192 0.0001. Control: water + glycerol (1:1), BA: benzoic acid (50 mM), ET: ethrel
193 (100 mM), K: potassium sulfate (500 mM), MeJa: methyl jasmonate (10; 25; 50
194 or 100 mM), LA: linolenic acid (200 mM), Iso: isoleucine (100 mM), MeJa + Iso:
195 methyl jasmonate + isoleucine (100 mM : 100 mM), SA: salicylic acid (14.5
196 mM).

197 **3.2. Individual variability of resinosis capacity**

198 When the individual resin production is plotted as a function of the
199 adjuvant paste used in the experiment with 1-year-old plants, it becomes

apparent that some individuals stand out, above or below the standard deviation in each treatment (Figure 2). LY and HY plants were excluded from the experiments carried out with 2- and 3-year-old saplings, in order to minimize the effects arising from the genetic variation. However, these contrasting extreme resin phenotype individuals were chosen for the gene expression studies (item 3.5).

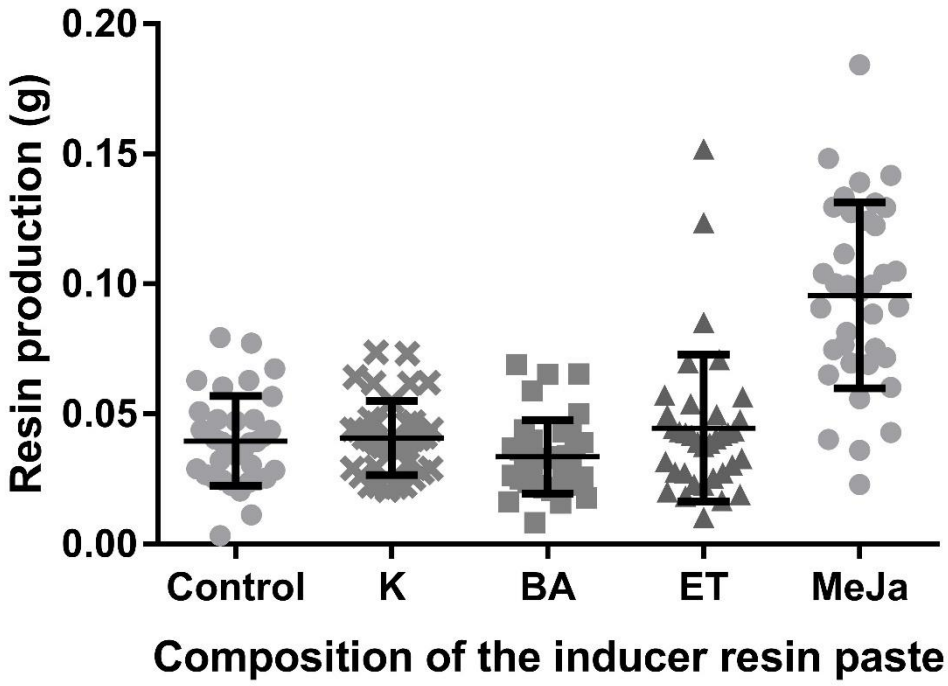


Figure 2. Resin production of individual 1-year-old seedlings of *P. elliottii* treated with different inducer paste composition (dots). Data shown in lines are means ± s.d. Control: water + glycerol (1:1), BA: benzoic acid (50 mM), ET: ethrel (100 mM), K: potassium sulfate (500 mM), MeJa: methyl jasmonate (100 mM).

3.3 Evaluation of point of injury in the stem, micropanel x brachyblast pick comparison, and MeJa concentration gradient on resinosis

Regarding the point of injury (basal, median or apical) evaluated in 1-year-old seedlings, most pastes induced a higher resin yield in the basal portion, while the apical portion exuded the least amount of resin (Figure 3). The median point was statistically similar to the apical point in all treatments, except for BA and MeJa, where it was also similar to the basal point. In the ET treatment, none of injury points showed statistical difference in relation to the others. For all treatments, except for MeJa, the apical, median and basal points did not differ statistically from the respective points in the negative control.

In 2-year-old saplings, negative control and ET had the same exudation pattern per stem portion observed for 1-year-old seedlings, whereas MeJa showed similar resin release for all stem parts (Figure 3). MeJa + Iso basal and median treated portions yielded statistically equivalent resin biomass, the apical segment producing less resin. All other treatments, but control basal versus apical portion, did not show differences among the three wounding points. Resin yield at the basal point was only statistically different from control for MeJa and MeJa + Iso treatments. For the median and apical points, however, all treatments except LA produced more resin than the control.

For 3-year-old trees, the drop of resin exudated from brachyblasts pick points showed statistical differences between all treatments containing different concentrations of MeJa in relation to the control, unlike that observed for the micropanel or total production, in which only concentrations of 50 and 100 mM differ statistically from the control. It was not possible to detect statistical difference between treatments MeJa 50 and MeJa 100 mM for micropanel or

brachyblast pick resin yield. Representative images of resin exuded in plants after the micropanel or brachyblast pick method are shown in Figures S4 and S5, respectively.

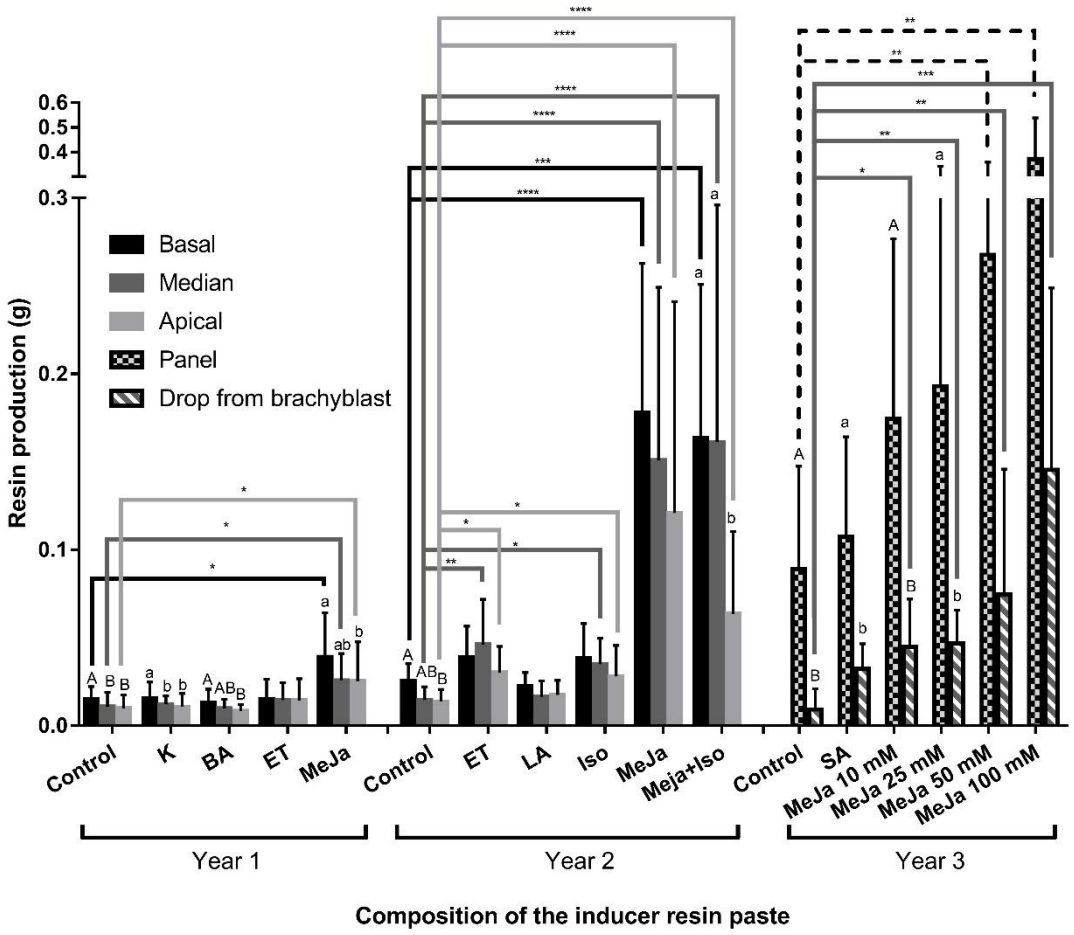
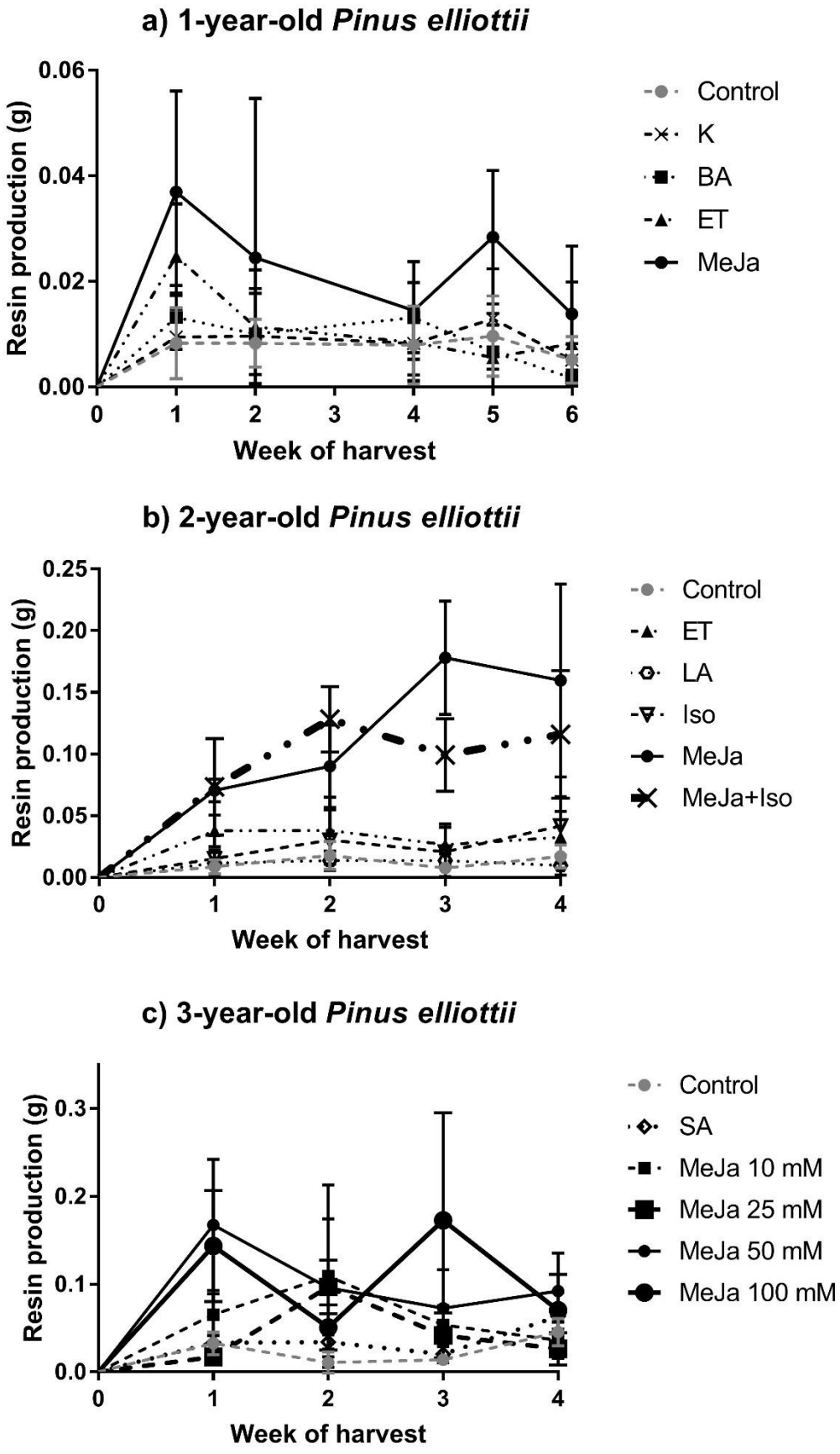


Figure 3. Effect of the stem portion of injury and stimulant paste composition on resin exuded from 1- or 2- year-old *P. elliottii* plants using the micropanel system. 3-year-old plants were only evaluated with basal micropanels and with the sum of brachyblast removed by the pick method at different parts of the plant. All analyzes are only valid for comparisons within each age. Data compared inside or between groups of stimulant paste types are shown as means ± s.d. Bars not sharing a letter within each stimulant paste treatment are significantly different by Dunn-test ($p < 0.05$). Connecting lines

and asterisks indicate statistical difference between the same point of injury in the different treatments in relation to the corresponding control, as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Control: water + glycerol (1:1), BA: benzoic acid (50 mM), ET: ethrel (100 mM), K: potassium sulfate (500 mM), MeJa: methyl jasmonate (10; 25; 50 or 100 mM), LA: linolenic acid (200 mM), Iso: isoleucine (100 mM), MeJa + Iso: methyl jasmonate + isoleucine (100 mM : 100 mM), SA: salicylic acid (14.5 mM).

3.4 Resinosis kinetics

For weekly kinetics of resin exudation, pastes containing MeJa and ET were more stimulant in the first week (Fig. 4a and b). However, in 1-year-old plants, there was a decrease in production in the second and third weeks (Figure 4a), whereas in 2-year-old plants the effect was more persistent (Figure 4b). In 3-year-old plants, the treatments MeJa 50 and MeJa 100 mM showed a higher initial stimulation of resinosis response, while MeJa 10 and MeJa 25 mM presented better stimulatory activity mostly at the second week, with paste reapplication (Figure 4c). The resumption of resin production upon paste reapplication was more evident for MeJa 100 mM in 2 and 3-year-old trees (Fig.4b and c), indicating that young plants respond to sequential stimulation in similar way as do their adult counterparts (Rodrigues-Corrêa and Fett-Neto, 2012)

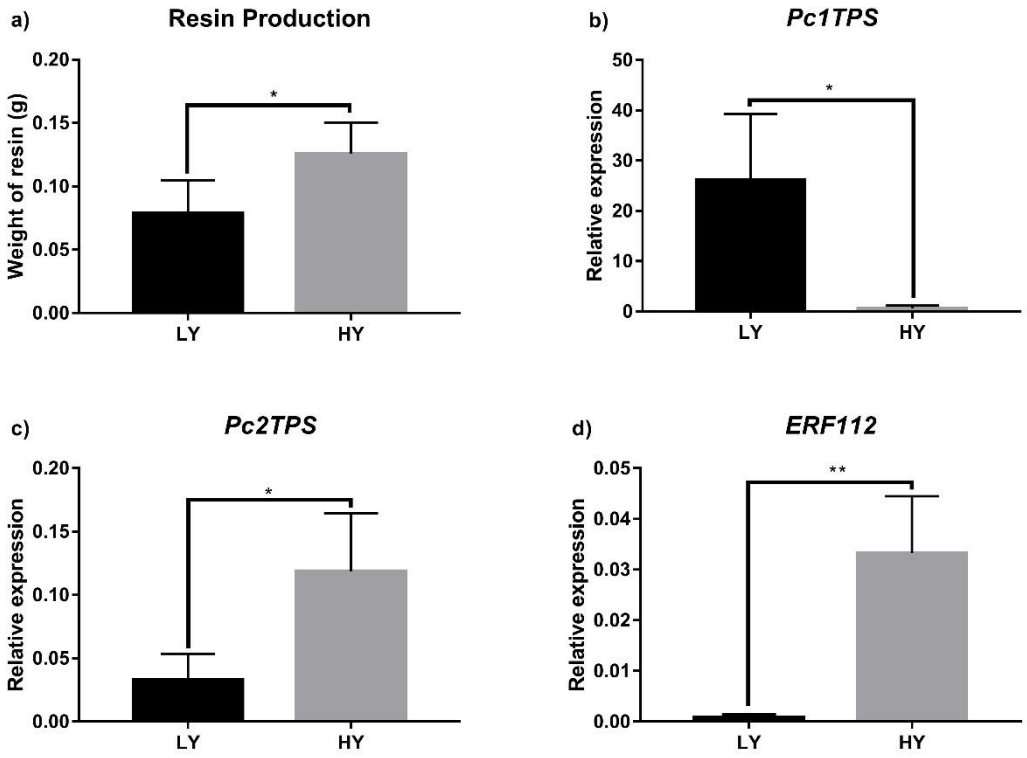

a) 1-year-old *Pinus elliottii*
Resin production (g)
0.06
0.04
0.02
0.00
0
1
2
3
4
5
6
Week of harvest
Control
K
BA
ET
MeJa
b) 2-year-old *Pinus elliottii*
Resin production (g)
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
Week of harvest
Control
ET
LA
Iso
MeJa
MeJa+Iso
c) 3-year-old *Pinus elliottii*
Resin production (g)
0.3
0.2
0.1
0.0
0
1
2
3
4
Week of harvest
Control
SA
MeJa 10 mM
MeJa 25 mM
MeJa 50 mM
MeJa 100 mM

270

Figure 4. Weekly kinetics of resin exuded from 1-, 2-, or 3-years-old *P. elliottii* plants submitted to different stimulant pastes using the micropanel method. Data shown as means ± s.d. The reapplication of paste occurred in the third week for 1-year plants (**a**) and at the second week for 2- or 3-year plants (**b** and **c**). Control: water + glycerol (1:1), BA: benzoic acid (50 mM), ET: ethrel (100 mM), K: potassium sulfate (500 mM), MeJa: methyl jasmonate (10; 25; 50 or 100 mM), LA: linolenic acid (200 mM), Iso: isoleucine (100 mM), MeJa + Iso: methyl jasmonate + isoleucine (100 mM : 100 mM), SA: salicylic acid (14.5 mM).

3.5 Gene expression in stimulated LY and HY individuals

The contrasting resin yield phenotypes of the LY and HY plants identified by the micropanel method at age of 1 year were confirmed at the age of 3 years (Figure 5a). The different genes examined related to terpene biosynthesis and regulation showed distinct expression profiles in LY and HY plants. *Pc1TPS* ((+)-α-pinene synthase) was more expressed in LY individuals (Figure 5b), whereas HY individuals had higher expression of *Pc2TPS* ((−)-β-pinene synthase) (Figure 5c) and of the ethylene responsive transcription factor *ERF112* (Figure 5d). In addition, *Pt3b* (alpha-farnesene synthase) was only amplified from LY (in all replicates), in contrast to *Pc3a* (abietadiene synthase), whose expression was only detected in HY (in 2 replicates only) (data not shown).



292

293 **Figure 5.** Resin production and gene expression in 3-year-old low (LY)
294 and high (HY) yield individuals stimulated with MeJa 50 mM after one week of
295 microtapping. **a**. fresh weight of resin exuded (g); **b**. to **d**. Relative expression of
296 genes *Pc1TPS* ((+)-α-pinene synthase) **b**, *Pc2TPS* ((−)-β-pinene synthase) **c**,
297 and *ERF112* (ethylene-responsive transcription factor) **d**. Bars represent mean
298 of the 4 individuals of each phenotype, with standard deviation denoted by the
299 lines above the bars. One or two asterisks indicate that the results were
300 significantly different ($p < 0.05$ and $p < 0.01$, respectively). Relative gene
301 expression in **b** through **d** was calculated with reference genes *HISTO3* and
302 *UBI*. Data were compared by unpaired t-test (LY: low-yield individuals, HY:
303 high-yield individuals).

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4. Discussion

4.1 Resin yield considerations

Experiments involving induction of terpene biosynthesis in young conifers with a focus on resin production remain scarce, but there are some reports of using young plants (up to two years-old) mainly for basic studies on defense responses. *Picea sitchensis* was used in comparative transcript profiling under herbivore attack (Ralph et al., 2006). The differential allocation of chemical defenses was observed in *Pinus radiata* (Moreira et al., 2012a), whereas the activation of wound signaling pathways through the exogenous application of MeJa or salicylic acid (SA) was evaluated in *Pinus pinaster* (Moreira et al., 2012b) and *Pinus banksiana* (Erbilgin and Colgan, 2012) seedlings. Other examples include the potential inducibility of terpene-based defenses associated with MeJa in juvenile *Picea abies* (Fäldt et al., 2003; Martin et al., 2003), *P. pinaster* (Moreira et al., 2009) and 17 other *Pinus* species (Carrillo-Gavilán et al., 2015). Quantification of resin production of *P. elliottii* plants as young as one year of age has been reported and shown to be stimulated by temporary flooding treatment (Ferreira et al., 2011).

The physiological role of sulfuric acid on promoting resinosis in adult pines is not known in detail. This chemical seems to act magnifying the wound and as a reactive oxygen species inducer, leading to a greater extension of the damage and consequent prolongation of resin exudation time (McReynolds and Kossuth, 1984; Rodrigues-Corrêa and Fett-Neto, 2012). In contrast to what was observed in seedlings of *Araucaria angustifolia* (Brazilian pine) (Perotti et al., 2015) sulfuric acid did not cause significant stimulation of resinosis in slash pine seedlings. This may reflect species-specific differences associated with the fact

that in Brazilian pine resin ducts are restricted to bark, whereas these secretory structures occur in both bark and sapwood of slash pine.

Moreover, plants of Brazilian pine used by Perotti et al. (2015) were four times older than the ones tested with the acid in the presently described experiments with slash pine. Similarly, the lack of response of SA in slash pine compared to Brazilian pine may be related to similar reasons, as well as to differences in concentration of the phenolic (approximately 3 times higher in the Brazilian pine experiments) (Perotti et al., 2015). Another aspect to consider is that whereas most of the present experiments with slash pine were carried out with the micropanel system, the study with Brazilian pine used removal of needles as wounding system, which may be relatively more responsive to stimulant adjuvants. Indeed, brachyblast pick tests with slash pine were more sensitive to MeJa, significantly increasing resin exudation even with application of lower concentrations of the oxylipin than the ones able to trigger a significant response in plants treated with the micropanel method (Figure 3c).

The average resin increment obtained by application of same concentration of MeJa in 1-year-old plants was 2.4-fold in relation to the control, increasing to 8.5-fold at 2-years and 5.3-fold at 3-years of age. These results were superior to those found by Heijari et al. (2008) in adult plants of *Pinus sylvestris*, when the increase in resin acids was 1.3-1.7-fold higher in MeJa-treated trees than in control trees, which may be related to species, age and organ treated, since the previous study examined sprayed needles and not wounded stems. On the other hand, the fold of resin exudation induction in MeJa-treated trees relative to the respective control trees found for 2 and 3-year

355 old slash pine trees was comparable to that previously observed for Brazilian
356 pine (Perotti et al., 2015).
357 Resin stimulant paste adjuvants that increased yield in adult slash pine
358 trees, such as BA, SA and K (Neis et al., 2018; Rodrigues-Corrêa and Fett-
359 Neto, 2013; Rodrigues et al., 2011), were not as effective in young plants
360 (Figures 1 to 3). This different response pattern may be at least partly explained
361 by the fact that young plants have more active basal metabolism than adult
362 plants, with a great investment in protein synthesis, growth and tissue formation
363 (Gershenzon, 1994), which could prevent major carbon allocation to resin.
364 Erbilgin and Colgan (2012) found that the concentration of constitutive
365 monoterpenes in phloem were higher in juveniles than in mature jack pine,
366 although the magnitude of induction was much larger in mature trees,
367 suggesting that ontogeny plays a role in jack pine defenses. It is also possible
368 that the complex network of resin ducts in adult trees may render them more
369 sensitive to stimulants than the relatively less developed and extended canals in
370 seedlings and saplings.
371 However, we found a significant increase in resin production using MeJa
372 in trees of the three ages examined and also with application of ET in 2-year-old
373 trees (Figure 3). This indicates that most potent adjuvants, as judged from adult
374 tree resinosis stimulation responses, are also perceived at early ages with
375 similar impact on resin exudation (Figures 1 to 3). In this sense, microtapping
376 may be used for faster, less costly and precocious selection of adjuvants with
377 potential high resin induction capacity in adult plants.
378 Overall, as plants aged in the present experiments resin exudation
379 increased (Figure 1). As plants age, they tend to proportionally increase the

amount of lignified tissues (Franceschi et al., 2005), photosynthetic capacity, and availability of energy reserves (Herms and Mattson, 1992; Wainhouse et al., 2005), which can affect the ability of inducing terpene biosynthesis (Erbilgin and Colgan, 2012). However, it is also necessary to consider the role of increased irradiance incidence in greenhouse (2 and 3 years old trees) versus growth room, allowing increased terpene biosynthesis (Jadaun et al., 2017). Moreover, pre-exposure to treatments in previous years may also favor higher basal resin yield level (Heijari et al., 2008).

While MeJa can act as volatile and vascular signal in plants and results in jasmonic acid biosynthesis, the active form of the molecule is a conjugate with the amino acid Iso (Berens et al., 2017; Thaler et al., 2012). The application of the isolated amino acid promoted higher resin yield than the negative control, and its response was similar to the ET application, suggesting it could be tested in adult plants. In paste containing equimolar concentrations of MeJa and Iso the response was different from the negative control, but statistically equal to that obtained only with MeJa application. Further studies need to be conducted, also at molecular level in young and adult plants, to elucidate the existence and mechanisms behind Iso-mediated induction of terpene biosynthesis.

Linolenic acid is the precursor of jasmonate, whose oxidation leads to activation of octadecanoid biosynthesis (Heil and Ton, 2008; Wasternack and Feussner, 2017), reason why it was tested in the present work. However, it was observed that this treatment produced the least amount of resin. This may be due to the synthesis of other types of defense molecules mediated by oxylipins (Babenko et al., 2017).

4.2 Evaluation of point of injury in the stem

The better resin yield of the basal portions of stems compared to more apical ones of 1-year old plants, as already discussed, may be due to the features of ontogenetically older, more lignified tissues that have higher resin production (Franceschi et al., 2005), unlike younger, actively growing apical portions with higher photosynthetic potential. Terpenoids and other constituents of secretory structures are synthesized in specialized and mostly non-photosynthetic cells (Lange, 2015), such as lower stem sapwood. The differences in resin yield of basal and apical stems of slash pine were no longer observed at 2 and 3 years of age (Figure 3), since tissue structure is more resembling of adult plants, perhaps showing higher competence for terpene induction. The fact that ET has induced resin production uniformly at the three points of the stem (Figure 3) may be explained by the fluidization and biosynthesis activation effect of this adjuvant on resin (McReynolds and Kossuth, 1984).

4.3 Resin exudation kinetics

Different aspects of time course of resin production have been studied in conifers, including *P. elliottii* (de Lima et al., 2016a; Popp et al., 1995; Rodrigues et al., 2008) and other species (Fäldt et al., 2003; Heijari et al., 2008; Martin et al., 2003; Miller et al., 2005). There is an overall pattern of increase in terpene-related transcript levels in the first days after treatment (wounding or MeJa), followed by an increase in the content of terpenes around day 15, returning to control levels by day 20 (references cited above). In the present study, in young plants resin yield peaks were observed as soon as in the first

week after application of the paste, decreasing in subsequent weeks and resuming production in the week following the second application (Figure 4).

Consistent with our observations, Heijari et al. (2008) cites that if MeJa is applied to the plants more than once it seems to increase the total defense level above that of untreated trees. This could be observed in young Scots pine needles, which presented a general increase in concentrations of monoterpenes 30 days after MeJa treatment. *P. elliottii* trees had an increase in monoterpene biosynthesis after 2 weeks of wounding (Popp et al., 1995), which is why wounds are periodically made for commercial resin production (Rodrigues et al., 2008). In our study, paste re-application promoted resinosis of plants that had already interrupted the exudation, even though no new injury had been caused. The persistence of MeJa signal is not expected to be long lasting, since it is a volatile signal, and easily degraded. Sixty days after the application of MeJa (5-100mM) on *P. pinaster* branches, there were no significant changes in resin content compared to the control (Moreira et al., 2009).

4.4 Early selection of high yield phenotypes and gene expression

The chemical defenses in conifers represent a high metabolic cost, with relevant ecological implications. In some cases, they are present in large concentrations throughout the plant, accounting for about 10% of tissue dry weight (Gershenzon, 1994; Moreira et al., 2014). Tree adaptation and survival in the field is often associated with the capacity of rapidly perceiving stress and increasing the concentration of chemical defenses (Schiebe et al., 2012). Conifer breeding has traditionally relied on phenotypic characterization of the breeding population near the harvest age to infer genetic quality for selection,

454 requiring up to 20 years to complete one breeding cycle (White and Carson,
455 2004). If combined with genomic selection the breeding cycle could be reduced
456 from 12–20 years to 4–7 years (Resende et al., 2012; Westbrook et al., 2015;
457 Westbrook et al., 2013). An interesting result from our work was the selection of
458 individuals with high potential of resin production based on the individual
459 distribution of resin per treatment at the first-year experiment (Figure 2).
460 The identification of individuals with extreme phenotypes of resin yield
461 (LY and HY) in the first year of age based on microtapping using the micropanel
462 system was further validated at the age of 3 years (Figure 5a), which showed
463 that the phenotype of 1-year-old seedlings was stable in subsequent years as
464 plants developed into saplings. Resin phenotype was associated with particular
465 resin biosynthesis-related gene expression profiles (Figure 5). Genes of pinene
466 synthases had opposite expression profiles, with *Pc2TPS* ((−)-β-pinene
467 synthase) showing higher expression relative to that of *Pc1TPS* ((+)-α-pinene
468 synthase) in HY plants, whereas the opposite was observed in LY individuals.
469 Interestingly, it has been detected that resin of adult slash pine individuals
470 followed the same chemical profile differences regarding the composition of
471 pinene isomers, with super-resinous trees showing a lower α-pinene/ β-pinene
472 ratio, and a higher ratio being recorded in low resin yield counterparts (Franciele
473 A. Neis, personal communication). Hence, it appears that overall pinene content
474 composition and related gene expression can be indicatives of resinosis
475 capacity in different individuals.
476 In addition, HY saplings yielded higher expression of the ethylene
477 responsive transcription factor *ERF112*. Ethylene plays a major role in the
478 response of plants to the most diverse arrays of biotic and abiotic stresses. In

conifer resin production, the gaseous phytohormone has been shown to enhance synthesis of phenolic compounds, sclereid lignification, and formation of traumatic resin ducts (Khan et al., 2017). The ethylene-responsive transcription factors are important in the signaling of stresses by specifically binding to GCC-box to control expression of genes induced by ethylene (Xu et al., 2007). Our results showing higher expression of an *ERF* in HY saplings are in good agreement with the findings that higher expression of this class of transcription factors was closely linked to resinosis in *Pinus massoniana* (Liu et al., 2015).

The meaning of *Pt3b* (alpha-farnesene synthase) exclusive amplification from LY and *Pc3a* (abietadiene synthase) from HY is not readily understood. Resin of slash pine is known for containing both the sesqui and the diterpene, and related genes were expressed in resin tapped adult trees (de Lima et al., 2016a). Relationships between resin chemical composition and yield phenotypes seem to vary between species (Karanikas et al., 2010; Liu et al., 2015). Further studies are needed to check for a possible difference in terpene composition between slash pine individuals with distinct resin yield phenotypes.

Microtapping can be an easy, quick and inexpensive tool for prospecting adjuvants with high impact on resin production. However, it has the limitation to discriminate only the increments that would be very high in adult plants, not presenting statistical differences for compositions already tested and proven useful in the field. In this sense, although some compositions that may have a significant effect on adult plants could pass unnoticed by microtapping-based screening tests, adjuvants that are effective in young plants will most probably

be validated in the field and potentially generate a significantly higher resin yield increase than that observed with commonly used pastes.

5. Conclusion

Selection of elite plants for terpenoid production in conifer stems and overall genetic improvement targeting resin yield are research topics demanding further efforts (Westbrook et al., 2013). Although it has been shown that it is possible to enhance terpenoid production with genetic approaches, ontogenetic and environmental factors can considerably influence resin yields (Neis et al., 2018; Rodrigues-Corrêa and Fett-Neto, 2013; Westbrook et al., 2013). The need to increase resin production with lower costs for the industry may be met by better strategies in the prospection of new stimulant pastes, the same applying for simple, fast and precocious selection of high resin yield individuals. The microtapping protocol (both based on micropanel and likely brachyblast pick methods) and possibly the yield-related gene expression marker profiles herein described represent valid strategies to achieve these goals. Future steps include testing putative diagnostic gene expression profiles in adult trees and following up on the stability of young-age identified resin-yield phenotypes into tree maturation after transfer to the field.

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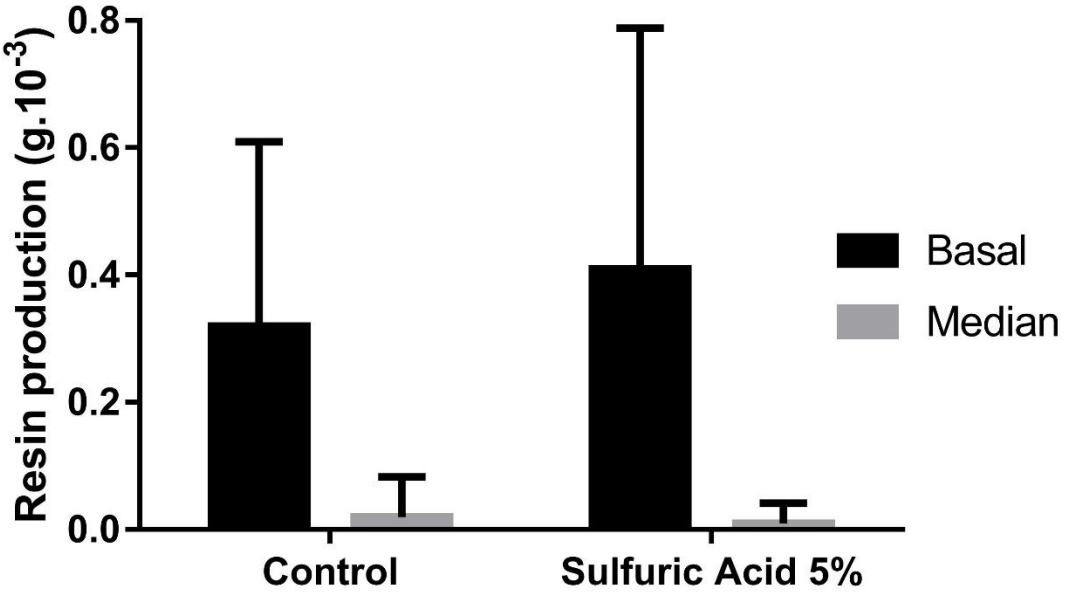
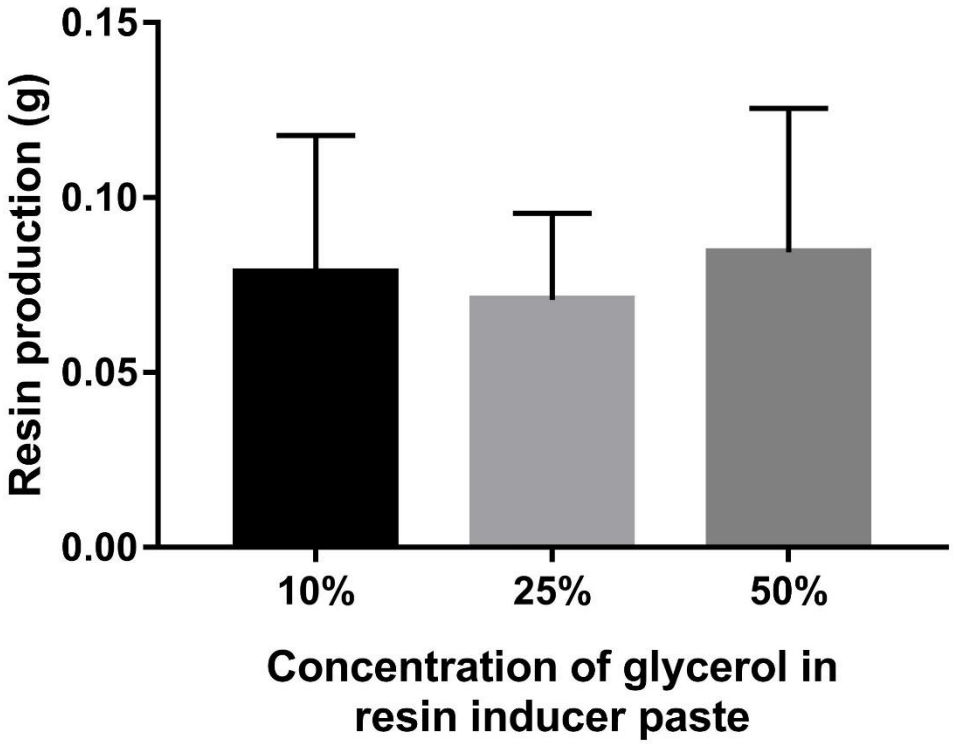
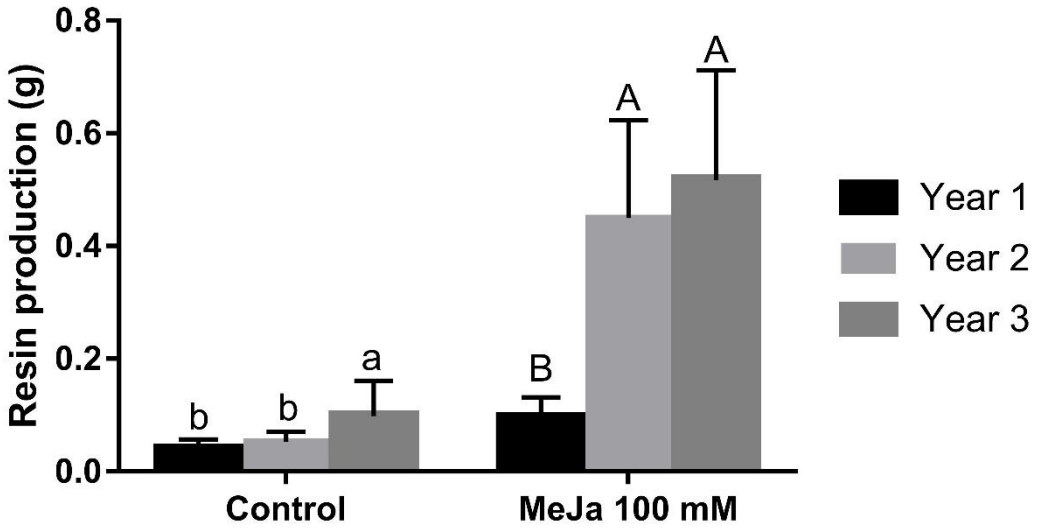
Supplementary Figures from "Resinosis of young slash pine (*Pinus elliottii* Engelm.) as a tool for resin stimulant paste development and high yield individual selection"



Supplementary Figure 1. Resin yield after one week in 10-month-old seedlings wounded in the basal and median portion of the stem by incision, followed by application of sulfuric acid at 5% (v/v) on the wound micropanel or not treated with any chemical (control). There is no significant difference between treatments at the same portion of the stem. Data is shown as means ± s.d.



750
751 **Supplementary Figure 2.** Resin yield after 2 weeks in 3-year-old plants
752 wounded in the basal portion of the stem by incision, followed by application
753 methyl jasmonate 50 mM on the wound micropanel with different concentrations
754 of glycerol as vehicle (v/v). Data shown as means ± s.d.
755



Supplementary Figure 3. Resin production at year 1, 2, and 3 of age in control individuals (wound only) and methyl jasmonate treated individuals (wound followed by 100mM MeJa application). Data shown as means ± s.d. Bars corresponding to the years not sharing a letter within each treatment are significantly different by Dunn-test [$p < 0.05$].

763
764
765 **Supplementary Figure 4.** Resin exuded from a micropanel in 2-year-old plants.

Supplementary Figure 5. Resin exuded from a brachyblast pick in 3-year-old plants.

VI. Capítulo 2.

Resin tapping transcriptome in adult slash pine (*Pinus elliottii* var. *elliottii*)

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Abstract

Conifers are armed with complex and dynamic chemical defenses against biotic agents, comprising synthesis of volatile terpenes and resin exudation. An elaborated mixture of terpenes, resin is one of the main non-wood forestry products, supporting chemical, pharmaceutical, food, and biofuel industries. Several efforts have been made to increase resin production in *Pinus elliottii* var. *elliottii* (slash pine), but the details of terpene biosynthesis regulation in this species are still poorly known. To better understand the molecular bases of resin production, the transcriptome of adult *P. elliottii* trees under field commercial resinosis was obtained using high-throughput sequencing technology. Samples were collected from the cambium after 5 and 15 days of treatment application, which included tapping followed by application of commercial resin stimulant paste or control wounding without paste application. Resin stimulant paste contained sulfuric acid and chloroethylphosphonic acid (Ethrel) - a known resin booster that releases ethylene, promoting responses to this hormone. Overall mean number of reads of all 16 libraries (2 treatments x 2 times x 4 replicated trees) was 34,582,048. Of these, 89% were mapped against the reference sequence, with a mismatch of 0.58%. Using the Blast2Go, 570 candidate genes were detected based on sequence annotation. By comparing the expression profile between paste and control, 310 differentially expressed genes (DEGs) were identified at 5 days, and 190 at 15 days with a significant fold change of $\log_2 > 1.2$. Regarding changes in time comparisons within each treatment, 210 and 105 DEGs were identified between 5 and 15 days within control and paste treatment, respectively. Among the genes with different expression patterns in the times and treatments examined were ethylene responsive transcription factors, geranylgeranyl diphosphate synthase, diterpene synthase, cytochrome P450 and ABC transporters, all of which may play important roles in resin production. In addition, several DEGs were annotated as MYB transcription factors, hormone response genes, such as auxin and jasmonate, and UDP-transferases, which may also be related to stem defense and damage repair mechanisms. Six genes were validated by RT-qPCR analysis and their expression patterns correlated well with the data obtained by RNAseq. This study represents the first transcriptomic investigation of resinosis of the main species used in the bioresin industry, whose genome still has not been sequenced. In addition, the work is the first case of molecular analyses of slash pine resinosis under field operational management. Such transcriptome data sheds light on the molecular bases of *P. elliottii* resin exudation in adult forests, with implications for stand management, stimulant paste development, genotype selection and breeding for high resinosis.

Key words: *Pinus elliotti*, resin, resinosis, transcriptome, adjuvant paste

1. Introduction

Conifers, the most advanced group of gymnosperms, have existed for over 300 Myr and thus have survived through periods of extreme changes in environmental conditions, competing plants, pest infestations and natural disasters (Warren *et al.*, 2015). Their adaptive success is largely due to the development of a defense system based on the synthesis and secretion of terpenes in all major organs and different tissues (Miller *et al.*, 2005; Hall *et al.*, 2013). Conifer resin is a viscous fluid composed of a complex and dynamic mixture of terpenoids such as monoterpenes, sesquiterpenes, and diterpenes (Zulak and Bohlmann, 2010) which is secreted from severed resin ducts when the tree is under biotic attack (Ralph *et al.*, 2006; Lange, 2015; Geisler *et al.*, 2016). Monoterpenes can inhibit herbivores and pathogens, diterpene resin acids act as physical barriers sealing wounds and trapping attacking insects, and sesquiterpenes have disruptive effects on insect development and antimicrobial activity (Schiebe *et al.*, 2012; Liu *et al.*, 2015).

Biosynthesis of terpenes in conifers starts from isomerization of two isoprenoid (C5) units, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). These molecules can be biosynthesized via two separate routes in plants, the methyl-erythritol 4-phosphate and mevalonate pathways. IPP is synthesized and isomerized to DMAPP by isopentenyl diphosphate isomerase, then prenyl transferases catalyze the condensation of these two C5-units to geranyl diphosphate (Pazouki and Niinemets, 2016). Their elongation to prenyl diphosphates with addition of IPP molecules leads to monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20), which are the substrates for terpene synthases (TPS) (Keeling and Bohlmann, 2006b).

TPSs comprise a large family of mechanistically related enzymes involved in both primary and secondary metabolism (Keeling and Bohlmann, 2006b). The events of evolutionary diversification and expansion of plant TPSs appear to be originated from gene duplications, domain losses, and sub- or neofunctionalizations, with subsequent divergence of an ancestral TPS gene of primary metabolism. This genetic plasticity seems to be a central player behind the chemical complexity of conifer specialized diterpenes (Hall *et al.*, 2013), since the modification of TPS products changes their physical properties and may alter their biological activities (Chen *et al.*, 2011). TPSs of high sequence identity even from closely related species may have different functions. In addition, even when lacking high sequence identity, TPSs of phylogenetically distant species can have, in an independent way, evolved same or resembling functions (Zerbe and Bohlmann, 2015). Furthermore, some of them produce multiple products whereas others produce only one compound (Chen *et al.*, 2011). The formation of diterpenoid resin acids involves further oxidation of diterpene synthase products by cytochrome P450-dependent monooxygenases (CYP450) (Keeling and Bohlmann, 2006a). The chemical composition of resin is dynamic and can change with environmental stresses to which the tree is exposed. Several TPS and CYP450 enzymes, their transcripts and products are regulated in response to insect attack or treatment with defense hormones (Zulak and Bohlmann, 2010).

Pine resin and its derivatives are used for production of solvents, adhesives, impermeable materials, paints, fragrances, food flavorings and in the pharmaceutical industry as precursors of active principles (Rodrigues *et al.*, 2013). The yield of resin is strongly correlated with tree diameter, radial resin

canal number and volume (Rodrigues *et al.*, 2008; Rodríguez-García *et al.*, 2014). New resiniferous ducts can be formed after bark injury (Nagy *et al.*, 2000; McKay, 2003). Resin chemical composition as well as resin induction capacity of each tree is highly variable, depending on genetic background (Westbrook *et al.*, 2013) and environmental factors, such as seasonality, abiotic and biotic stresses (Rodrigues-Corrêa and Fett-Neto, 2013). Commercially, exudated resin is obtained from transverse wounds mechanically inflicted to the trunk of adult trees (bark stripping), often at two-week intervals (Rodrigues *et al.*, 2013). Besides mechanical wounding, exudation can be affected by external factors such as abiotic stress and application of chemical stimulants (Moreira *et al.*, 2012; Rodríguez-García *et al.*, 2015). Under treatment with chemical elicitors, such as sulfuric acid and the ethylene precursor 2-chloroethylphosphonic acid (Ethrel), resin production is stimulated (Rodrigues and Fett-Neto, 2009).

Although the use of resin stimulant pastes containing different chemical agents in *Pinus elliottii* Engelm var. *elliottii* (slash pine) has been extensively studied (Rodrigues *et al.*, 2008; Rodrigues and Fett-Neto, 2009; Rodrigues *et al.*, 2011; Rodrigues *et al.*, 2013; Neis *et al*., 2018), the molecular bases involved in signal transduction triggered by these adjuvants are still unclear. At the same time, in recent years sequencing technology has developed rapidly and transcriptome analysis has become a promising tool for qualitative and quantitative analysis of gene transcripts in many non-model plants, including gigagenome trees, as is the case of pinus (de la Torre *et al.*, 2014; Wei *et al.*, 2015).

914 This work aimed to expand understanding on the molecular mechanisms
915 involved on resin induction as well as the effects of applying resin stimulant
916 paste containing an ethylene precursor on tapped trees under field conditions.
917 For this, a transcriptome dataset was generated to explore the profile of
918 resinosis in *P. elliottii* using the Illumina HiSeq™ 2500 platform. Functional
919 annotation, comparative analysis and KEGG pathways of subset transcripts
920 revealed significant expression changes in control and treated trees at both
921 times analyzed after wounding. Transcript abundance patterns provide a
922 valuable genetic resource to better understand the molecular and physiological
923 mechanisms involved in resin induction, as well as a tool for management of
924 forest stands and genetic improvement.
925
926 **2. Methodology**
927 **2.1. Plant material**
928 In this study we used 16-year-old trees of slash pine that met the criterion
929 of diameter at breast height ranging from 65 to 90 cm. The experiments were
930 conducted at Irani Celulose forest installations at Balneário Pinhal, RS, Brazil
931 (30◦14′S and 50◦14′W). Resin tapping and stimulant paste application were
932 performed following commercial practices on forest industry (Rodrigues *et al.*,
933 2011). Four randomly trees located in the inner part of the forest that were
934 previously tapped for resin production biweekly during one year with
935 conventional stimulant paste were exposed for each treatment. On the wound
936 was applied an adjuvant paste composed of 3 % of 2-chloroethylphosphonic
937 acid (v/v) (Ethrel), a synthetic precursor of ethylene, and 20 % of sulfuric acid
938 (v/v). To confer viscosity, the adjuvant compounds were dissolved in aqueous

939 solution and rice husk powder as an inert substrate (Fuller *et al.*, 2016). Paste
940 treatment, from now on referred to as Et, corresponded to directly applying a
941 streak of stimulant paste on the wounded bark right after stripping. Control
942 treatment was bark stripping only, without paste application, and is hereafter
943 referred to as C. Gene expression analyses were done with samples collected
944 after 5 and 15 days in a novel mini bark streak slightly above the wound zone,
945 since the wound line produces poor quality RNA (de Lima *et al.*, 2016a; de Lima
946 *et al.*, 2016b).

947 **2.2. RNA Extraction and Sequencing**

948 Total RNA of the sixteen samples grouped into four libraries (Control 5
949 days - C5d, Control 15 days - C15d, Adjuvant paste 5 days - Et5d, and Adjuvant
950 paste 15 days - Et15d, all of them from each of 4 trees, which corresponded to
951 independent biological replicates) was extracted from vascular cambium of
952 slash pine plants using *PureLink RNA Kit* (Thermo Fisher), as previously
953 described (de Lima *et al.*, 2016b). Nanodrop spectrophotometer (Thermo
954 Scientific) was used to quantify RNA concentration. Samples were precipitated
955 with 3 M sodium acetate, 5 $mg.ml^{-1}$ glycogen and ethanol. Pellets were kept in
956 70 % ethanol for shipping. RNAseq sequencing was performed by Fasteris S.A.
957 (Switzerland). Library preparation and RNA sequencing were performed with
958 Fasteris S.A. standard protocols (single-end 1x 125 bp Illumina HiSeq High-
959 Output) after depletion of rRNA with RiboZero kit.

960 **2.3. Data filtering and mapping of reads**

961 Raw reads were filtered before data analysis. Identification of low-quality
962 sequences, empty sequences and sequences with only one copy was done
963 using FastQC software. Based on these data, the Trimmomatic software

version 0.32 (Bolger *et al.*, 2014) was used to eliminate sequences of reads with a Phred quality score below 30, as well as the adapters. The filtered reads were then anchored to the reference genome of *Pinus taeda* (https://treegenesdb.org/Drupal) with HISAT2 2.0.4 (Pertea *et al.*, 2016) . Transcript abundance estimation in RPKM values and subsequent differential expression analysis were performed using the Cufflinks program version 2.1.1.

2.4. Differentially expressed genes (DEGs) and annotation analysis

Genes were considered differentially expressed between combined comparisons of two libraries when statistical analyses indicated both q-value < 0.05 and $|\log_2(\text{Fold Change, FC})| > 1.2$. Treatment effect responses were considered as those in which the difference in gene expression was observed at the same time of harvest with different stimulation procedures (C5d*x*Et5d and C15d*x*Et15d) and time responses were those in which the same treatment was evaluated at different harvest times (C5d*x*C15d and Et5d*x*Et15d). Transcript sequences assembled by Cufflinks were assigned to GO terms through the Blast2GO software (Conesa and Götz, 2008).

Analyses of metabolism category of each differentially expressed gene were performed using MAPMAN (Usadel *et al.*, 2009) and the KEGG database (Kanehisa and Goto, 2000). In these analyses, a Blastn search was performed using the sequences of DEGs as queries against the *Arabidopsis thaliana* genome with a cutoff e-value of $1.e^{-10}$. Each one of the best hits was retrieved and its metabolic annotation data (either from KEGG or MAPMAN) were assigned to its query. The enriched GO terms were then visualized using ReviGO and the network was edited using Cytoscape (Shannon *et al.*, 2003; Supek *et al.*, 2011). The Multiple Array Viewer program was used for gene

clusters within each comparison, using Hierarchical clustering, taking the distance metric through Pearson correlation. Since *Pinus elliottii* genome has not been sequenced to date, all genes identified in this study are in fact “like-genes”, which, for the sake of simplification will be referred to by their putative name.

2.5. Isolation of RNA and RT-qPCR

Six genes involved in defense mechanisms and terpene biosynthesis were chosen for confirmation of RNA-seq data via RT-qPCR (highlighted as ǂ in the tables S2 and S3). This selection included a 9-lipoxygenase (9-LOX; PITA_000001587), an ethylene response transcription factor (ERF112; PITA_000040911), a geranylgeranyl diphosphate synthase gene (GGDS; PITA_000026252), two terpene synthase genes (diterpene synthase, DTS; PITA_000000451, and taxadienol acetyl transferase, TAT; PITA_000030936) and a cytochrome P450 monooxygenase gene (CYP736B; PITA_000090075). Primers were based on the amplified sequences in the RNA-seq alignment with at least 6 species of related trees, whose sequences were obtained using Phytozome version 12.1.6 (https://phytozome.jgi.doe.gov) and aligned with Genomatix (http://www.genomatix.de). Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table S1). Total RNA (500 ng) of each one of at least three different trees was treated with 1U DNAse I (Life Technologies) before cDNA synthesis. Total RNA concentration was determined using UV spectrophotometer (Nanodrop, Thermo Scientific). 500 ng of RNA was treated with 1U DNAse I and DNAse I reaction buffer, according manufacture instructions (Thermo Fisher). For cDNA synthesis, the treated RNA was reverse

transcribed using oligo-dT primers and M-MLV reverse transcriptase (Life Technologies) in a total volume of 20 µl.

All analyses were performed using three biological replicates for each time point and stimulation treatment. Quantitative PCR reactions were carried out in a technical quadruplicate for each sample and performed in fast optical 96-well reaction plates (MicroAmp™ Applied Biosystems) using a StepOne™ Real-Time PCR System (Applied Biosystems). The reaction mix for each sample was composed by 10 µl of 10-fold diluted sample cDNA, 4.25 µl of sterile Mili-Q water, 2 µl of 10× PCR Buffer (Invitrogen), 1.2 µl of 25 mM $MgCl_2$ (Invitrogen), 0.2 µl of each of the 10 µM forward and reverse gene-specific primers, 0.1 µl of 10 mM dNTP (Invitrogen), 2 µl of SYBR Green (1:10,000, Molecular Probes, Applied Biosystems), and 0.05 µl Platinum® Taq DNA polymerase (5 $U.\mu l^{-1}$ Invitrogen) (de Almeida *et al.*, 2010). The reactions have been set to include an initial denaturation at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, 10 s at 60 °C, and 15 s at 72 °C. After that, samples were held at 40 °C for 2 min for annealing and then heated from 55 to 99 °C with a ramp of 0.5 $°C.s^{-1}$ to generate the melting curve of the amplified products and validate product specificity. Obtained data were analyzed by the comparative Cq (quantitative cycle method) (Livak and Schmittgen, 2001). The PCR efficiency from the exponential phase (Eff) (Ramakers *et al.*, 2003) was calculated for each individual amplification plot using the LinReg software. The average of PCR efficiency for each amplicon was determined for each plate and used in further calculations. The reference genes HISTO3 and UBI were used for all analyses (de Lima *et al.*, 2016a). Statistical evaluation was performed after normal distribution analysis and data were submitted to one-tailed not-

paired t-test (Goni *et al.*, 2009) using the software Graphpad Prisma 7. Pearson correlation analysis was carried out with qPCR and RNAseq data for the genes of interest.

2.6. Extraction and analyses of monoterpenes

Resin samples of 5 individuals of each treatment condition were harvested for analyses of major terpenes. In a vial, approximately 80 mg of resin were solubilized in 3 ml diethyl ether and then kept in an ultrasonic bath at room temperature for 20 min (Rodrigues *et al.*, 2011). The purification of samples was done by a solid-phase extraction using Extractclean silica columns (Altech Inc., Columbia, MD, USA; 500 mg.8.0 ml^{-1}). For qualitative analyses, 1 µl of the solutions was injected into a gas chromatograph (GC-2010) at 220 °C with auto-injector (Autosampler AOC-20i) connected to a mass spectrometer (Model GCMS-QP2010S) (Wang *et al.*, 1997). It was used a column 95 % polydimethylsiloxane : 5% phenyl model Restek Rtx 5MS (30 m x 0.25 mm x 0.25 µm; Restek Corp., Bellefonte, PA, USA). The sequence protocol of running started with initial temperature 40 °C for 2 min and increase at a rate of 5 $°C.min^{-1}$ until 200 °C (1 min hold), and an additional ramp of 10 $°C.min^{-1}$ up to 300 °C, kept for 5 min. As carrier gas it was used Helium at a constant flow of 1.02 $ml.min^{-1}$ and linear velocity of 36.5 $cm.s^{-1}$ (Perotti *et al.*, 2015). Compound identification was based on comparison of retention indices from a series of n-alkanes and mass spectra from literature data or that we performed with authentic samples (Rodrigues *et al.*, 2011). Data were expressed as average percentual of total terpenes in resin of five individual trees evaluated. To address the differences in chemical composition under treatments or time course after wounding, data were submitted to ANOVA followed by Tukey tests.

In every case, $p \leq 0.05$ was used. Tests were run using the software Graphpad Prisma 7.

3. Results

3.1. Raw sequencing data and mapping of RNA-seq reads to Pinus genome

Illumina HiSeq2500 high-throughput sequencing generated reads with an average length of 120.6 nucleotides. There were 35,940,121 single-end reads for C5d, 35,092,676 for C15d, 33,551,956 for Et5d and 33,743,440 for Et15d. Read quality by trimmomatic package showed Phred Quality Score (Q30) average of 94.7%. Reads were mapped to the *Pinus taeda* genome using HISAT2 and the alignment summary showed an average of 30,747,200 input reads mapped to the genome, which corresponds to approximately 89% of mapped reads, with mismatch of 0.58%.

3.2. Identification of differentially expressed genes

The mapping output generated by HISAT2 was processed by Cufflinks toolkits for transcript assembly and differential gene expression analysis. To narrow down the DEG list, genes with $|\log_2(FC)| > 1.2$ were selected for further investigation resulting in a total of 570 differentially expressed genes (Fig. S1). This analysis provided 310 DEGs for the comparison C5d*x*Et5d, 190 for C15d*x*Et15d, 201 for C5d*x*C15d and 105 for Et5d*x*Et15d (Fig. 1). Overall, comparisons of treatments and times of exposure showed higher number of up-regulated than down-regulated DEGs, except for C5d*x*C15d, in which the numbers of up and down-regulated genes were similar (Fig. 1). A Venn diagram showing the genes expressed at different experimental comparison was

prepared to illustrate the distribution of DEGs in the libraries C5d*x*Et5d, C15d*x*Et15d, C5d*x*C15d and Et5d*x*Et15d (Fig. S1).

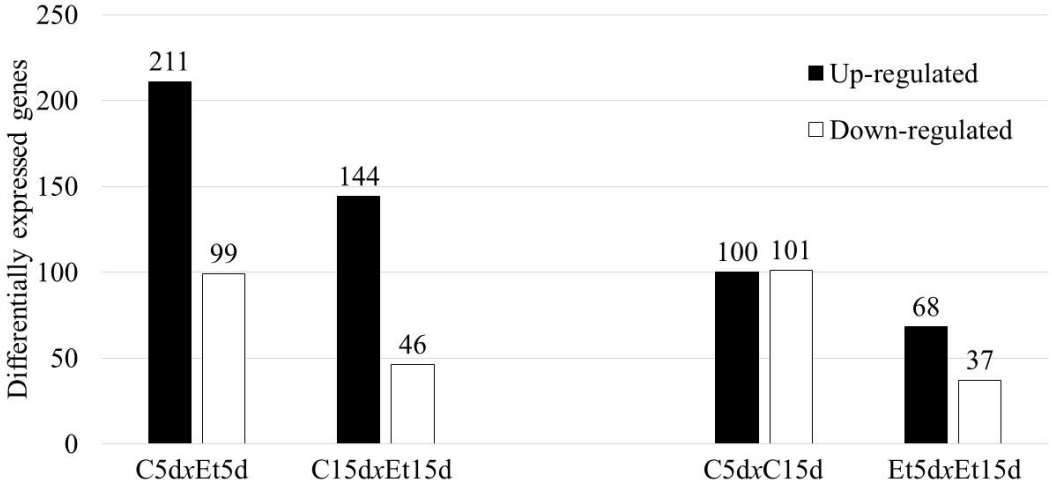


Figure 1. Number of differentially expressed genes in slash pine trees under resinose treatments: Control 5 days - C5d, Control 15 days - C15d, Paste treatment 5 days - Et5d, and Paste treatment 15 days - Et15d. The differentially expressed genes were identified with the criteria $|\log_2(FC)| > 1.2$ and $p < 0.001$. DEGs classified in each comparison according the expression profile: black bars represent up-regulated genes and white bars the down-regulated.

The annotation and gene ontology yielded various functional classes of genes. Well represented functions included stress response factors, ethylene responsive factors, transferases, mechanisms of cell wall regeneration, and secondary metabolism. Among up-regulated genes of the 121 DEGs in the comparisons involving treatment effect (C5d*x*Et5d and C15d*x*Et15d), there were mainly elements associated with cell wall synthesis, MYB type transcription factors, response to ethylene transcription factors, chitinases, transferases, enzymes linked to nucleic acids, such as ubiquitin, polymerases, tRNA, and histones, in addition to genes annotated as transposons related to retroviruses.

A DEG annotated as expansin showed induction in the comparison C5d*x*Et5d and repression in C15d*x*Et15d (Fig. 2a). Its expression increased right after the injury, with induction in the presence of paste, but decreased in expression over time, which was also intensified with the stimulation treatment. When comparisons between times after exposure to treatment were evaluated, only 25 DEGs proved similar between harvest times (C5d*x*C15d and Et5d*x*Et15d). Sequences annotated as terpene synthase or related to abscisic acid (ABA) were found among induced DEGs, whereas among repressed genes, annotations like endoglucanase and GGDP-synthase appeared (Fig. 2b). Tables 1 and 2 show the DEGs that are similar for comparisons in Figures 2a and 2b, respectively (those presented at intersection of Venn diagram), and its expression pattern. Table S2 lists the DEGs in each library present outside the intersection between the comparisons for both treatment or time effect, shown in Figures 2a and 2b.

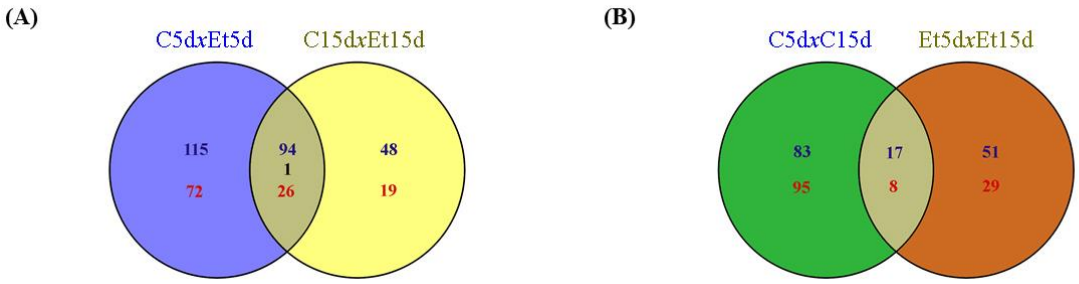


Figure 2. Chart of common DEGs in comparison of libraries. Venn diagram showing the comparative analysis of the **A)** treatment effect (C5d*x*Et5d and C15d*x*Et15d libraries) or **B)** time effect (C5d*x*C15d and Et5d*x*Et15d). Blue numbers represent up-regulated genes and red numbers correspond to down-regulated genes. In Figure 2.A, the black number at the junction of the two groups for treatment effect (paste application) represents a gene which showed distinct expression pattern for each of the comparisons (PITA_000032006, an expansin-

B3-like, that was up-regulated for C5d*x*Et5d and down-regulated for C15d*x*Et15d).

The C5d*x*Et5d comparison allowed the identification of a chloroplast phenylalanine hydroxylase and a probable linoleate 9S-lipoxygenase. Genes involved in auxin response and its metabolism, MYB type transcription factors and cytochrome P450 monooxygenase enzymes were observed in the C15d*x*ET15d comparison. Regarding time after treatment, DEGs annotated as enzymes involved in the biosynthesis of gibberellin (GA) were observed in the comparison between the control libraries (C5d*x*C15d), whereas jasmonate O-methyltransferase was recorded in the Et5*x*Et15d comparison.

3.3. Gene ontology (GO) enrichment analysis of differentially expressed genes

DEGs with $|\log_2(FC)| > 1.2$ in each comparison were considered to carry out GO enrichment analysis. The relation of GO terms annotated as biological process, cellular component and molecular function was determined (Fig. 3a and 3b). For biological processes, there was an abundance of DEGS annotated as biosynthetic processes, secondary metabolism and DNA integration in all four comparisons analyzed. For cellular component, the largest number of annotations was in membrane component, whereas for molecular function the four comparisons exhibited DEGs annotated as ion binding, oxidoreductase activity, binding to nucleic acids or to DNA. Correlations of some of the metabolic processes and GO terms were used to generate a network interactive analysis (Fig. S2a-c and Fig. S3a-c). Key GO terms, such as cell-wall organization, signal transduction, energetic processes, stress, oxireductase activity, immune response, defense response, cell morphogenesis, and

chemical stimulus, played critical role in the network regulation of differentially expressed GO terms (Fig. S2a and S3a). Analysis of cellular components highlighted nuclear activity, as well as membranes and energy-related organelles (Fig. S2b and S3b). Regarding molecular functions, nuclease activity, ATPase related activities, protein signaling, and enzyme activity, including redox-related ones, were predominant (Fig. S2c and S3c).

Table 1. List of similar DEGs observed in adjuvant paste treatment effect analyzes (C5d*x*Et5d and C15d*x*Et15d; represented by the Venn diagram in Figure 2.a). The expression pattern found in each comparison is represented by colors intensity (up-regulated in blue and down-regulated in red).

Gene	log_2(FC) C5d*x*Et5d	log_2(FC) C15d*x*Et15d	Description
PITA_000077568	+inf	+inf	xyloglucan endotransglucosylase hydrolase 9-like
PITA_000058756	6,98351	5,42983	peroxidase 64
PITA_000065557	6,33952	6,6443	ACT domain-containing ACR4-like
PITAhm_002677	5,73728	5,70878	Retrovirus-related Pol poly from transposon TNT 1-94
PITAhm_000499	5,70547	3,51961	Gag-Pol poly
PITA_000082902	5,63723	4,21724	transmembrane 45B-like
PITAhm_000219	5,47224	3,90059	PREDICTED: uncharacterized protein LOC105963074
PITA_000020711	5,46927	5,66672	early nodulin-93-like
PITA_000070233	5,31027	5,93039	NAC transcription factor 25
PITA_000068210	5,1363	4,46216	RING U-box superfamily
PITA_000062048	4,81595	4,47889	extradiol ring-cleavage dioxygenase-like
5A_all_VO_L_2_T_232453/409051|m.39590	4,77943	6,56141	multi-copper oxidase type I family
PITA_000022694	4,76329	4,46361	unknown
PITAhm_002526	4,63014	5,81663	isocitrate lyase
2A_I2_VO_L_1815_T_162/162|m.25252	4,56232	2,26719	chaperone dnaJ chloroplastic-like
PITA_000023255	4,54533	3,70162	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000015934	4,3684	4,28037	flavonol synthase flavanone 3-hydroxylase-like
PITA_000044665	4,36339	3,71475	aldehyde oxidase GLOX-like
PITA_000083219	4,07023	4,74147	methylesterase 10-like
PITA_000065353	3,95809	2,77141	unknown
PITA_000040403	3,87248	4,03852	(R)-mandelonitrile lyase-like
PITA_000032006	3,84083	-2,67021	expansin-B3-like
PITA_000066910	3,82634	2,7298	Enzymatic poly
PITA_000074704	3,77918	4,98951	cysteine protease partial

PITA_000059888	3,70852	2,87229	MKS1-like
PITA_000014344	3,70111	2,54169	probable prolyl 4-hydroxylase 3
PITA_000088525	3,6957	3,93894	Germin subfamily 1 member 1
PITA_000021167	3,67469	4,79362	hyoscyamine 6-dioxygenase-like
PITAhm_001040	3,60389	4,2001	Retrovirus-related Pol poly LINE-1
1A_I5_VO_L_6720_T_12/20|m.10219	3,5555	3,83401	1-aminocyclopropane-1-carboxylate oxidase 5-like
PITA_000050738	3,50391	2,91467	Pectinesterase pectinesterase inhibitor 3
PITAhm_003121	3,46395	3,03775	DETOXIFICATION 16-like
PITA_000064486	3,3257	3,17307	plant cysteine oxidase 3-like isoform X1
PITA_000001688	3,32394	4,54449	nac transcription factor 56
PITAhm_002408	3,28561	2,98397	DNA RNA polymerases superfamily
PITA_000040911	3,27799	3,69935	Ethylene-responsive transcription factor ERF112
PITA_000067469	3,23905	3,71355	rust resistance kinase Lr10-like
PITAhm_000270	3,19329	3,35955	gag-pol poly
5A_I12_VO_L_1830_T_43/53|m.43615	3,17989	2,42949	class VII chitinase
PITA_000018064	3,15598	4,58488	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000016691	3,14487	4,33877	oxidoreductase family
PITA_000062362	3,12088	4,0009	metalloendo ase 2-MMP
PITAhm_002285	3,106	3,13339	hypothetical protein VITISV_037667
PITA_000042337	3,09456	4,0916	cyanogenic beta-glucosidase-like
PITA_000033647	3,03186	2,36055	dnaJ homolog subfamily B member 6 isoform X1
PITA_000011698	2,95956	2,90637	UDP-glycosyltransferase 86A1 isoform X2
PITAhm_000498	2,91039	3,41271	retrotransposon Ty3-gypsy subclass
PITA_000015926	2,89188	2,47282	transposon Tf2-1 poly isoform X1
6A_I27_NT_comp35812_c0_seq12|m.61587	2,68097	3,46121	alcohol dehydrogenase partial
PITA_000079952	2,64517	2,75822	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000021466	2,61344	3,69465	NAC domain-containing 68-like

PITA_000000751	2,57818	2,759	PREDICTED: uncharacterized protein LOC107459471
PITAhm_002745	2,56693	3,36116	transposon Ty3-G Gag-Pol poly
PITA_000067596	2,5373	3,30126	methylesterase chloroplastic
PITA_000001345	2,53189	2,79298	arogenate dehydrogenase chloroplastic-like
PITA_000026782	2,51141	3,96609	tyrosine aminotransferase-like
PITAhm_000280	2,51117	2,03502	Gag-Pol poly
PITA_000064319	2,47773	3,17939	glucan endo-1,3-beta- basic isoform
PITA_000013524	2,46769	2,11109	#N/D
PITA_000063560	2,45663	2,35758	E3 ubiquitin- ligase PUB23-like
PITA_000019354	2,42418	3,80946	#N/D
PITA_000048284	2,32895	5,31747	DMR6-LIKE OXYGENASE 1-like
PITA_000052757	2,30375	1,76384	GRAM-containing ABA-responsive partial
PITA_000045152	2,29073	3,51862	geraniol 8-hydroxylase-like
PITA_000045571	2,24907	2,67272	lactosylceramide 4-alpha-galactosyltransferase-like
6A_I27_NT_comp35025_c0_seq3|m.61527	2,24654	2,25901	probable LRR receptor-like serine threonine- kinase At3g47570
PITAhm_002576	2,21438	3,19185	shikimate O-hydroxycinnamoyltransferase-like
5A_all_VO_L_2_T_176474/409051|m.39255	2,21361	1,94689	---NA---
PITA_000012909	2,18357	3,71385	unknown
PITA_000009191	2,16996	2,35843	cytochrome P450 82C4-like
PITAhm_001055	2,16555	2,37848	PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC101508457
PITA_000076202	2,1373	2,91168	transcription factor HBP-1b(c38)-like
PITAhm_001334	2,07349	1,91283	gag-pol poly
PITA_000016145	2,04668	3,80146	PREDICTED: uncharacterized protein LOC104880764
PITA_000070498	2,01349	1,90097	Glutamate decarboxylase 1
PITA_000002797	1,93936	2,45479	Retrovirus-related Pol poly from transposon TNT 1-94
PITAhm_003079	1,92633	1,97594	transposon Tf2-1 poly

PITA_000082905	1,88515	1,58966	plant cysteine oxidase 2-like
PITA_000020650	1,84023	2,25871	LINE-1 reverse transcriptase like
PITA_000057601	1,82791	2,78975	receptor kinase HSL1
PITA_000011595	1,81069	1,33859	acid phosphatase 1-like
PITA_000026640	1,80309	2,42027	probable WRKY transcription factor 31
PITAhm_001974	1,79033	1,61673	sucrose synthase partial
PITA_000008251	1,77699	2,79488	unknown
PITA_000002298	1,77641	2,24574	WRKY transcription factor 18-like isoform X1
1A_I5_NT_comp37310_c0_seq4|m.8665	1,76365	2,88795	GDSL esterase lipase At4g26790
PITA_000010421	1,74492	2,00406	gag-pol poly
PITA_000012357	1,70328	2,53473	#N/D
PITA_000092677	1,69314	3,31094	clavaminate synthase At3g21360
PITA_000014100	1,61109	1,9434	NRT1 PTR FAMILY -like
PITA_000030235	1,5232	2,33092	#N/D
PITA_000063119	1,51099	1,46746	LOB domain-containing 25-like
PITA_000017343	1,47863	1,69957	transcription repressor MYB5-like
PITAhm_001302	1,47238	2,16813	#N/D
PITA_000051181	1,34437	2,48299	Retrovirus-related Pol poly from transposon TNT 1-94
PITAhm_002875	-1,70033	-2,67546	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000050820	-1,76931	-1,73173	retrotransposon unclassified
PITAhm_003358	-1,92172	-1,47681	gag-pol poly
PITA_000018993	-2,02497	-1,86617	GDSL esterase lipase At4g01130
PITA_000021442	-2,03851	-1,4116	probable L-type lectin-domain containing receptor kinase
PITA_000078953	-2,32147	-3,85398	LOB domain 41
PITA_000012858	-2,36754	-2,9746	cinnamoyl- reductase 1
PITA_000093229	-2,56942	-2,10395	gamma-glutamyl peptidase 5-like
5A_I13_VO_L_57_T_465/508|m.44157	-2,70093	-1,78572	tau class glutathione S-transferase

PITA_000007408	-2,79586	-1,94575	pectate lyase
PITAhm_000578	-3,13201	-2,72465	retrotransposon Ty3-gypsy subclass
PITA_000045010	-3,36103	-2,50979	AAA-ATPase At4g30250-like
PITA_000095401	-3,55198	-3,50698	cysteine ase 15A
PITA_000008452	-3,67292	-3,29655	dirigent pDIR2
PITA_000044045	-3,721	-4,82165	BAHD acyltransferase DCR
PITA_000003292	-3,89855	-2,48041	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000053347	-4,06802	-4,87815	beta 1,3 glucanase
PITA_000024978	-4,22777	-2,64079	amino acid permease 3-like
PITA_000022627	-4,42047	-2,85774	tRNA modification GTPase
5A_I15_VO_L_1_T_27897/41278|m.46048	-5,01809	-3,54183	geraniol 8-hydroxylase-like
PITA_000007362	-5,05967	-2,94717	laccase
PITA_000025831	-5,75329	-4,72625	L-ascorbate oxidase-like
PITA_000058385	-6,97992	-2,61778	cytochrome P450 CYP736A12-like
PITAhm_003135	-inf	-inf	Retrovirus-related Pol poly from transposon TNT 1-94
PITA_000004858	-inf	-inf	Retrovirus-related Pol poly from transposon TNT 1-94
1A_all_VO_L_14184_T_8/12|m.2915	-inf	-inf	transcription factor bHLH118-like

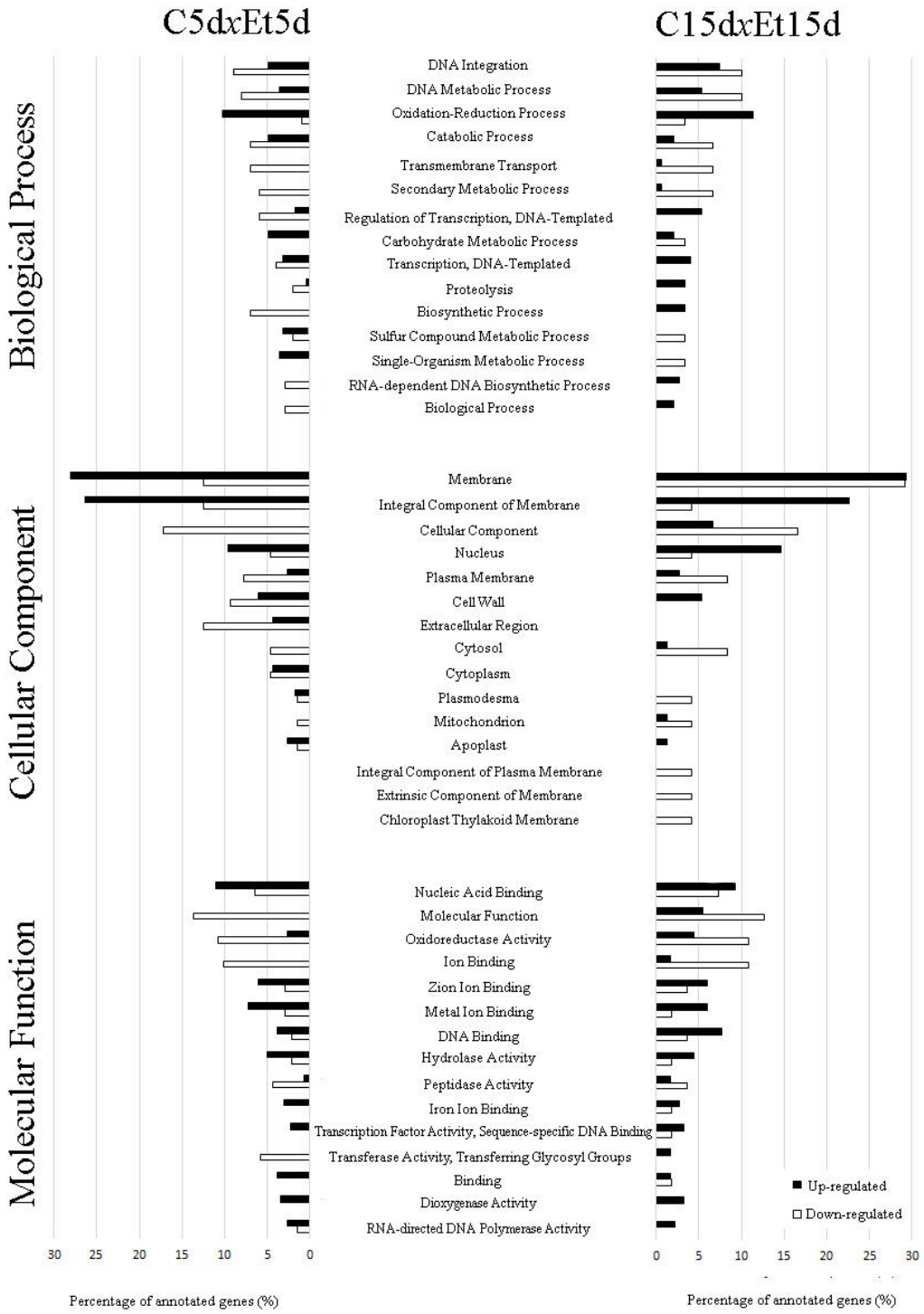
Table 2. List of similar DEGs observed in time effect analyzes (C5d*x*C15d and Et5d*x*Et15d; represented by the Venn diagram in Figure 2.b). The expression pattern found in each comparison is represented by colors intensity (up-regulated in blue and down-regulated in red).

Gene	log_2(FC) C5d*x*C15d	log_2(FC) Et5d*x*Et15d	Description
5A_all_VO_L_4895_T_4/35|m.41012	+inf	+inf	kDa proline-rich
PITAhm_001260	3,51316	5,80744	Gag-Pol poly
PITA_000005361	2,42918	3,89894	aspartyl protease family At5g10770-like
PITA_000022063	2,60474	3,56991	WALLS ARE THIN 1
PITA_000072764	2,1524	3,0841	pectinesterase 31
PITA_000004115	2,23172	2,89408	related to ABI3 VP1 2

PITA_000083984	2,27646	2,75924	probable ascorbate-specific transmembrane electron transporter1
PITA_000056712	4,58095	2,73052	unknown
PITA_000074179	1,73385	2,71918	probable aspartyl protease At4g16563
PITAhm_001856	1,76588	2,6899	hypothetical protein PRUPE_ppa019964mg, partial
PITA_000010789	1,86604	2,57645	unknown
PITA_000003940	2,13363	2,44951	12-oxophytodienoate reductase 11
PITA_000003222	2,11056	2,2471	cinnamoyl- reductase 1-like
PITA_000000451	2,42264	2,22817	monofunctional diterpene synthase
PITA_000040347	2,25063	2,07826	abscisic acid 8 -hydroxylase 1
PITA_000060448	3,15275	2,04103	beta-amylase 7-like isoform X1
PITAhm_001462	1,60957	2,03829	unknown
PITA_000019299	-1,52013	-1,64533	beta-carotene hydroxylase 1
PITA_000008252	-2,43201	-1,89613	gag-pol poly
PITA_000043460	-1,97647	-2,11526	hypothetical protein 0_12117_01, partial
PITA_000010966	-3,78137	-2,22164	ferritin- chloroplastic
PITA_000026252	-3,48688	-2,39507	geranylgeranyl diphosphate synthase
PITA_000019355	-2,10293	-2,42021	hypothetical protein VITISV_035196
PITA_000064319	-3,44398	-2,74231	glucan endo-1,3-beta- basic isoform
PITA_000078953	-1,88782	-3,42034	LOB domain 41

1168
1169

A.


C5dxEt5d
C15dxEt15d
Biological Process
Cellular Component
Molecular Function
DNA Integration
DNA Metabolic Process
Oxidation-Reduction Process
Catabolic Process
Transmembrane Transport
Secondary Metabolic Process
Regulation of Transcription, DNA-Templated
Carbohydrate Metabolic Process
Transcription, DNA-Templated
Proteolysis
Biosynthetic Process
Sulfur Compound Metabolic Process
Single-Organism Metabolic Process
RNA-dependent DNA Biosynthetic Process
Biological Process
Membrane
Integral Component of Membrane
Cellular Component
Nucleus
Plasma Membrane
Cell Wall
Extracellular Region
Cytosol
Cytoplasm
Plasmodesma
Mitochondrion
Apoplast
Integral Component of Plasma Membrane
Extrinsic Component of Membrane
Chloroplast Thylakoid Membrane
Nucleic Acid Binding
Molecular Function
Oxidoreductase Activity
Ion Binding
Zion Ion Binding
Metal Ion Binding
DNA Binding
Hydrolase Activity
Peptidase Activity
Iron Ion Binding
Transcription Factor Activity, Sequence-specific DNA Binding
Transferase Activity, Transferring Glycosyl Groups
Binding
Dioxygenase Activity
RNA-directed DNA Polymerase Activity
Up-regulated
Down-regulated
30 25 20 15 10 5 0
0 5 10 15 20 25 30
Percentage of annotated genes (%)
Percentage of annotated genes (%)

B.

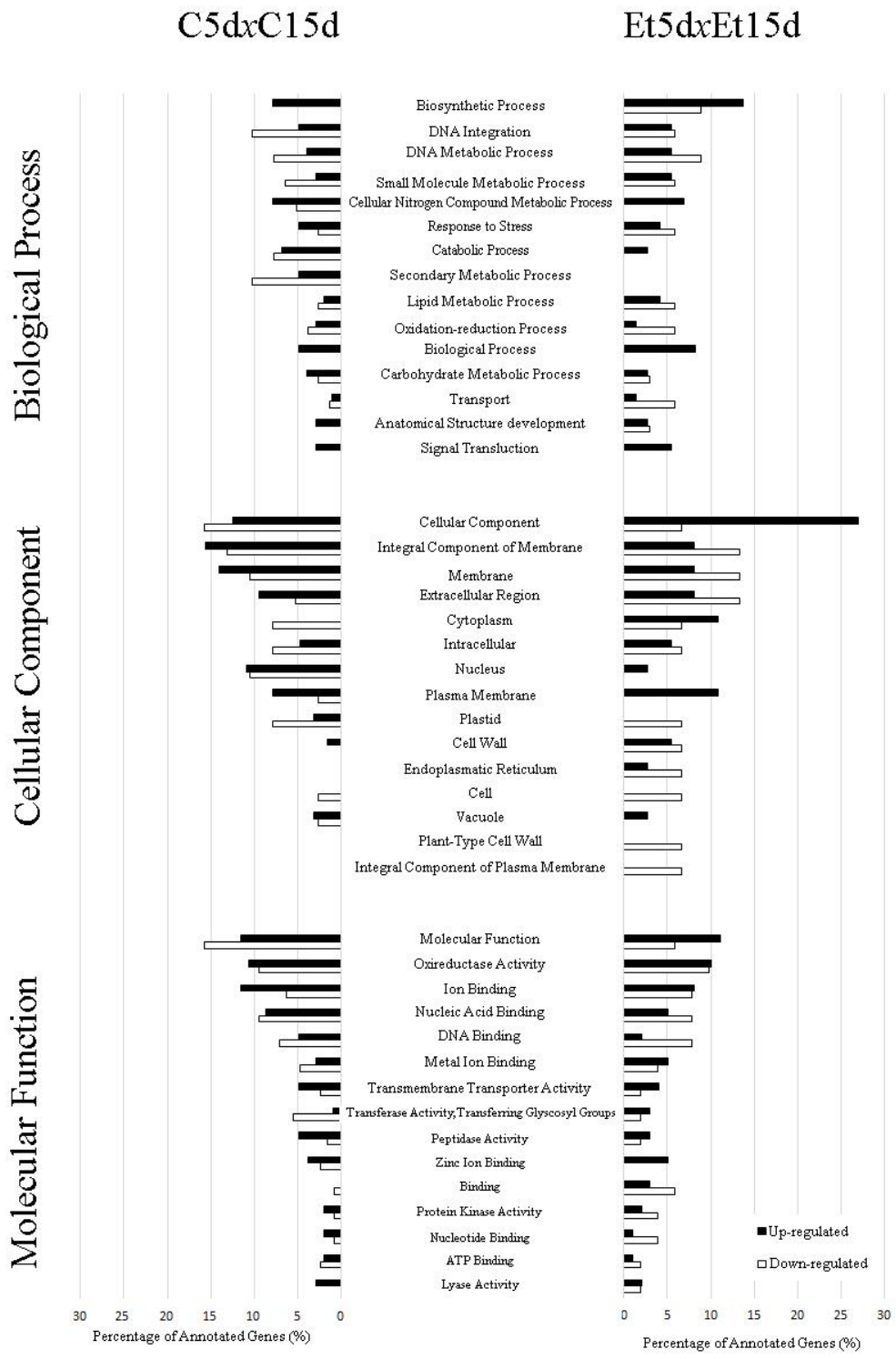


Figure 3. GO classification analysis of differentially expressed genes. DEGs were annotated in three main categories: biological process, cellular component, and molecular function. **A)** treatment effect (C5d*x*Et5d and C15d*x*Et15d libraries) and

B) time course (C5d*x*C15d and Et5d*x*Et15d). The x-axis indicates the percentage of a specific category of DEG in that main category. Black bars represent up-regulated genes and white bars the down-regulated.

3.4. Validation of RNA-seq data by quantitative real time PCR (RT-qPCR)

To validate the data from RNA-seq analysis, six genes related to terpene biosynthesis or stress response were selected for expression analysis by RT-qPCR. The expression data of these selected genes in both RNA-seq and RT-qPCR analyses are shown in Figure 4. In general, there was a strong correlation between these two sets of data, which was shown by a linear relationship for gene expression (Fig. S4). The overall value of $R^2 = 0.7748$ and $p = 0.008927$ according to Pearson's correlation analysis between the fold-change of the two methods, suggests that the RT-qPCR and RNA-seq data exhibited consistent agreement in all upregulated and downregulated genes evaluated.

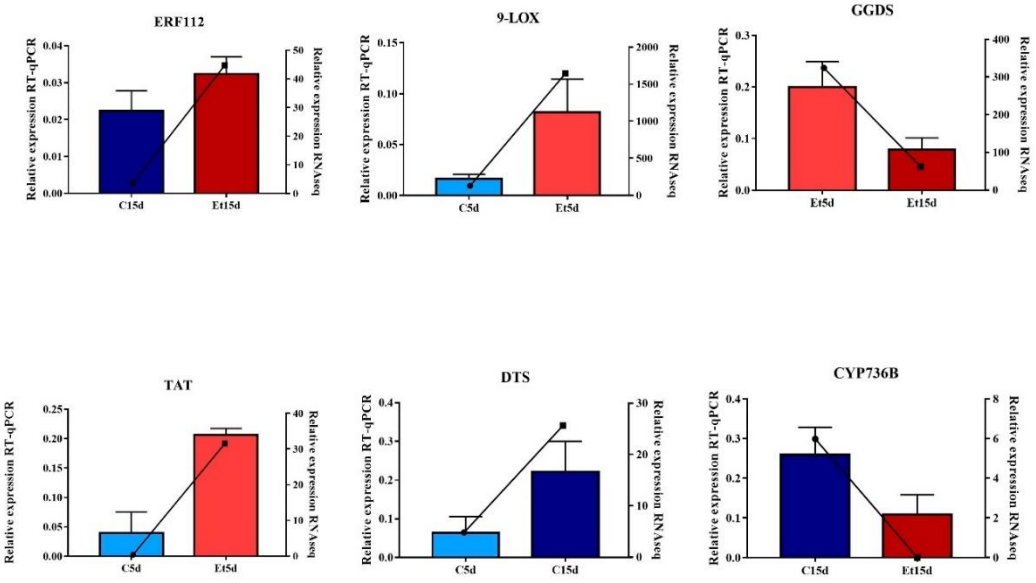


Figure 4. Quantitative RT-PCR validation of tag-mapped candidate genes associated with the response to damage, defense signaling and yield of

oleoresin. Relative expression levels of qRT-PCR calculated using Histone 3 and Ubiquitin as internal control are shown as columns in the left y-axis. Relative expression levels of transcriptomic analyses (RNAseq) are represented as lines and shown in the right y-axis. Blue bars represent control treatment and red bars represent treatment with adjuvant paste. Light bars represent collection data on day 5 and dark bars represent collection on day 15. Data shown as means ± standard error of the mean (s.e.m.) for one-tailed not-paired t-test. ERF112: ethylene-responsive transcription factor ERF112 (p = 0.1199); 9-LOX: probable linoleate 9S-lipoxygenase 5 (p = 0.05809); GGDS: geranylgeranyl diphosphate synthase (p = 0.04121); TAT: taxadienol acetyl transferase partial (p = 0.07406); DTS: monofunctional diterpene synthase (p = 0.07495); CYP736B: cytochrome P450 monooxygenase (p = 0.07604).

3.5. Chemical composition of resin

Proportion of the four major components of *Pinus* resin was evaluated by GC-MS. Composition of resin terpenes changed over time during induction, with β-pinene being the major component at 15 days after bark injury, independent of treatment, while at 5 days the most abundant component was α-pinene (Fig. 5). The increase of β-pinene was opposite to the variation of limonene, whose concentration decreased during the time evaluated.

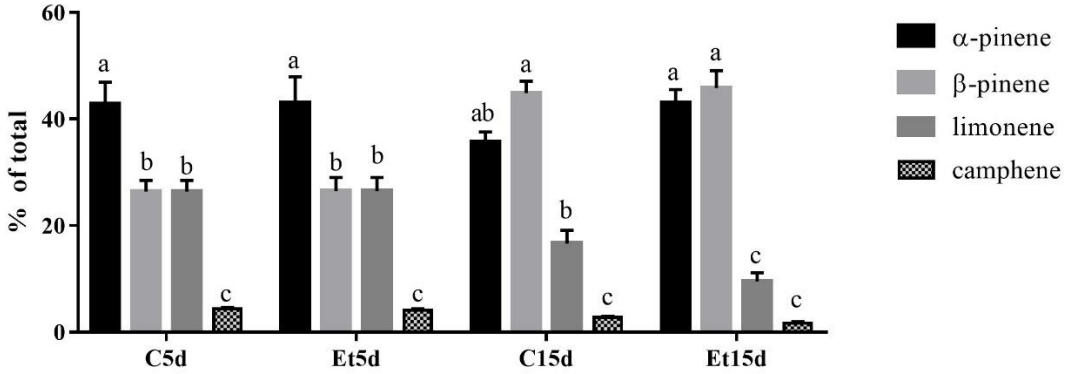


Figure 5. Monoterpene quantification. Concentration of main monoterpenes in slash pine resin at day 5 or 15 after tapping produced by control or paste treated

trees, expressed on % of weight. Bars indicated by different letters are statistically different at $p < 0.05$ by simple ANOVA followed by Tukey's multiple comparisons test. Data shown as means ± s.e.m.

4. Discussion

Terpenoid-based defenses comprise advanced biochemistry to produce elaborated metabolites of significant cost to the tree (Gershenzon, 1994). Resin induction in commercial forests has been explored through the application of resinosis inducing agents, mainly based on molecules that increase damage (e.g. sulfuric acid), phytohormones, such as ethylene, jasmonate, salicylic acid and auxin, or enzymatic cofactors and activators of enzymes involved in the synthesis of terpenes (Martin *et al.*, 2003; Rodrigues and Fett-Neto, 2009; Rodrigues *et al.*, 2011; Perotti *et al.*, 2015; Neis *et al*., 2018). However, the molecular mechanisms involved in the induction of resinosis by adjuvant pastes or in response to tapping damage remain unclear, especially in field trees derived from seeds, which are genetically variable and represent most of the pine forest stands currently explored for resin around the world.

4.1 Behavior of DEGs related to biosynthesis (TPS and CYP450s) and transport (ABC) of resin

Conifers produce large quantities of resin composed of complex mixtures of dozens of different terpenoids in response to injury or insect attack. The first committed reaction in diterpenoid biosynthesis is catalyzed by terpene synthases (TPSs), while cytochrome P450 (CYP450)-dependent enzymes catalyze functional modifications of diterpene scaffolds, such as oxygenation and carboxylation reactions (Chen *et al.*, 2011). The expression of TPSs and

CYPs can be induced by defense responses (Martin *et al.*, 2003; Martin *et al.*, 2004; Zulak and Bohlmann, 2010).

Increased expression of a gene annotated as taxadienol acetyl partial transferase (PITA_000030936, Table S3) in response to treatment at 5 days (C5d*x*Et5d) (Fig. 4), and a monofunctional diterpene synthase (PITA_000000451, Table S3) in comparisons of time points, both under control and treatment condition (C5d*x*C15d and Et5d*x*Et15d) (Fig. 4), were recorded. Gene expression of β-pinene synthase, α-pinene synthase and levopimaradiene/abietadiene synthase had already been tested for the same samples by RT-qPCR in one of our previous studies (de Lima *et al.*, 2016a). In these analyses, β-pinene synthase showed increased expression for time point evaluation under treatment with adjuvant paste (Et5d*x*Et15d) and the comparison of treatment effect at 15 days (C15d*x*Et15d). On the other hand, α-pinene synthase showed difference in expression only in the comparison of time under the effect of stimulating paste (Et5d*x*Et15d), with up-regulated profile, whereas levopimaradiene / abietadiene synthase showed no significant differences in expression in any treatment comparison or time point. Stimulated resinosis treatments were consistent with increased expression of some of the genes examined (de Lima *et al.*, 2016a), although the absence of widespread gene expression changes may be explained by the existence of several post-transcriptional levels of resin production regulation and the specific time needed between gene transcription and the production of metabolites.

CYP450s are heme-dependent oxidases with a plethora of functions, including insertion of oxygen into substrates, generating new molecules involved in both primary and secondary metabolism, such as lignin intermediate

biosynthesis, phytohormones, sterols, terpenes, flavonoids, isoflavonoids, and furanocoumarins (Schuler, 1996). Terpenoid metabolism includes phytohormones, photosynthetic pigments, plant defense compounds, electron carriers and structural membrane components, involving a large number of CYP450s (Hamberger *et al.*, 2011; Weitzel and Simonsen, 2013; Geisler *et al.*, 2016). Most CYP450 genes are highly regulated, being able to respond to phytohormones, light radiation damage, metal toxicity, mechanical injury, water availability, ions, salinity, temperature and pathogens (Cheng *et al.*, 2010). In conifers, CYP450s diversify the chemical nature of resin terpenes. Accordingly, several CYP450s were differentially expressed in the present study. Specifically, it is worth highlighting CYP720B2 (PITA_000006872), involved in synthesis of diterpene resin acids (Hamberger *et al.*, 2011; Geisler *et al.*, 2016), which was down-regulated in C5d*x*Et5d and up-regulated in Et5d*x*Et15d (Table S2). In addition, CYP736B/CYP736A (5A_I15_VO_L_2583_T_52/67|m.47001), a predominantly gymnosperm subfamily, but which may be related to resistance to fungal infection in grapevine (Cheng *et al.*, 2010; Warren *et al.*, 2015), was up-regulated in Et5d*x*Et15d and down-regulated in C5d*x*Et5d and C15d*x*Et15d (Table S2).

Expression of a gene annotated as ABC transporter (ABC transporter G family member 36-like isoform X2, PITA_000066825) had varied behavior, being repressed in C5d*x*Et5d but induced in Et5d*x*Et15d (Table S2). The ABCs (ATP-binding cassette transporters) are proteins involved in special metabolite transport, such as flavonoids, anthocyanins and terpenes. In conifers, ABC transporters are involved in transport of resin terpenes and the expression of ABC transporter genes is associated with resin production in loblolly and

masson pine (*Pinus massoniana* Lamb) (Westbrook *et al.*, 2013; Liu *et al.*, 2015).

4.2 DEGs related to hormones

Ethylene is a simple gaseous phytohormone participating in cell size regulation, often restricting elongation. It is most involved in processes such as fruit ripening, senescence, and abscission, with multiple roles in regulation of metabolism at cellular, molecular, and whole plant level (Khan *et al.*, 2017). Ethylene interacts with other phytohormones (such as auxins and methyl jasmonate) and is involved in the response to biotic and abiotic stresses. In Pinaceae, ethylene induces resin synthesis and the differentiation of resin canals (Hudgins and Franceschi, 2004), and ethylene responsive transcription factors (ERFs) are critical for resin biosynthesis (Liu *et al.*, 2015). In accordance, ERF112 (PITA_000040911) was found to be up-regulated under the effect of resin stimulant paste in both times evaluated (C5d*x*Et5d and C15d*x*Et15d) (Table S3).

At 15 days post wounding, a pronounced expression of auxin-induced genes (auxin-induced 15A-like and xyloglucan endotransglucosylase hydrolase 9-like, PITA_000028753 and PITA_000077568, respectively) (Li *et al.*, 1994; Catalá *et al.*, 1997) was noted in treatments containing stimulant paste (Table S3). Auxin may be involved in the formation of stem structures, radial growth of wood and resin canals in conifers. In poplar, auxin-responsive genes in wood-forming tissues react dynamically to changes in cellular indole-3-acetic acid (IAA) levels (Moyle *et al.*, 2002; Nilsson *et al.*, 2008). The application of Ethrel to *P. halepensis* seedlings promoted the differentiation of longitudinal resin ducts (Yamamoto and Kozlowski, 1987). In conifers, ethylene induces chemical

defenses against insects and pathogens (Hudgins and Franceschi, 2004), both modulating synthesis of defense compounds and via crosstalk between IAA and the ACC-oxidase, involved in ethylene biosynthesis. This effect can induce the formation of resin canals and becomes even more pronounced in the periodic resination, as is the case of the trees used in this experiment, since auxin effects in resin yield are mostly perceived in the long run (Rodrigues *et al.*, 2011).

Moreover, other genes involved in auxin regulation have also been identified. In the comparison C15d*x*Et15d, the adjuvant paste led to increased expression of genes related to auxin homeostasis via conjugation, such as indole-3-acetate O-methyltransferase (PITA_000065724, Table S2). The methyl ester of IAA, product of this enzyme, may be involved in the development of multiple biological processes in *Picea glauca*, including embryogenesis and xylem formation (Zhao *et al.*, 2009). The concentration of active IAA available can be regulated at different levels, including biosynthesis, conjugation/deconjugation and degradation. Methylation is a mechanism of IAA modulation, since MeIAA is not an active auxin and is more nonpolar compared to IAA, so it faces less restriction in diffusing across membranes. There is evidence that methyl-IAA can be converted back to IAA by the action of an esterase (Yang *et al.*, 2008). Thus it is possible that MeIAA may represent a relevant form of IAA transport to neighboring cells or distant targets (Korasick *et al.*, 2013).

A GGDS (PITAhm_003208) and an *ent*-kaurenoic acid oxidase (PITA_000060831) were up-regulated in the comparison Et5d*x*Et15d (Table S2 and S3). These enzymes may be related to both terpene metabolism and GA

biosynthesis (Zi *et al.*, 2014). In the C5d*x*C15d comparison, expression of a gene encoding the enzyme GA_{20}-oxidase (PITA_000012007) was repressed (Table S3). This is a key enzyme in GA metabolism since it catalyzes the penultimate step in GA biosynthesis, producing GA_9 and GA_{20}, which are then converted to biologically active GA molecules by GA_3-oxidases (Oikawa *et al.*, 2004). GA promotes growth and simultaneously affects lignification and wood formation. GA can stimulate secondary growth and xylem differentiation in many plant species such as *Arabidopsis*, potato, poplar and hybrid aspen (Biemelt *et al.*, 2004; Jeon *et al.*, 2016). The interaction between GA and ethylene is dynamic and complex, with positive or negative effects. Ethylene inhibits growth in a GA-antagonistic manner, but, depending on the developmental process and environmental condition, ethylene may favor GA synthesis (Weiss and Ori, 2007). GA has been shown to induce expression of monoterpene synthases in sage and essential oil concentration increase and decrease was observed with increasing levels of GAs and blocked GA biosynthesis, respectively (Schmiderer *et al.*, 2010). In poplar overexpressing PdGA20ox1, a gene encoding a GA_{20} oxidase, defense signaling genes were downregulated in stem tissues, suggesting an energetic competition between growth and defense (Park *et al.*, 2015). Taken together, this information suggests that the repression of GA_{20}-oxidase observed in our data in the comparison C5d*x*C15d (Table S3) may reflect an investment in defense signaling and response.

In Et5d*x*Et15d we found a reduction in the expression of jasmonate O-methyltransferase gene (PITA_000085027) (Table S2), whose product is an enzyme related to the production of methyl jasmonate, an airborne and vascular signal of plant defense (Seo *et al.*, 2001). In conifers, induction of defense

response involves jasmonate production, which is mediated by ethylene and results in traumatic resin duct differentiation (Aloni, 2013). This profile is expected considering that wounding response starts to occur almost immediately, extending itself for the first days followed by a subsequent decrease.

4.3 Other defense compounds

The increased expression of a probable linoleate 9S-lipoxygenase (PITA_000001587) in the comparison C5d*x*Et5d (Table S2) is consistent with defense mechanisms induced by disruption of cell membranes, peroxidation of unsaturated fatty acids and oxylipin production by lipoxygenases (LOX). Although this DEG cannot be unequivocally related to the synthesis of defense compounds in *Pinus*, evidence supports that oxylipins derived from 9-LOX can be stimulated during defense responses in plants (Marmey *et al.*, 2007; Saubeau *et al.*, 2013; Babenko *et al.*, 2017). Jasmonate is mainly the result of linoleic acid peroxidation with involvement of 13-LOX, essential for wound-induced defense. 9-LOX-derived oxylipins, although less studied, may also respond to stress signals and take part in defense mechanisms (Saubeau *et al.*, 2013). In tobacco and potato, there are reports of 9-hydroperoxy polyunsaturated fatty acids accumulation after damage by pathogens. In potato tuber, the lipoperoxides derived from LOX can also stimulate morphogenesis either directly via JA formation or indirectly through modulation of IAA levels (Lim *et al.*, 2017). During cotton hypersensitive reaction to bacterial pathogen, lipidic peroxidation dependent of 9-LOX was concomitant with emergence of hypersensitive symptoms and water loss of infected tissues (Marmey *et al.*, 2007).

The phenylpropanoid pathway comprises the branches of lignin and flavonoid biosynthesis, as well as many other specialized metabolites that are important for conifer defense, often sharing common regulatory proteins, precursors and enzymes (Warren *et al.*, 2015). Transcription factors of MYB type (MYBTFs) may regulate several aspects of plant secondary metabolism and associated specialized cell development across diverse plant species (Bedon *et al*, 2007; Bedon *et al*., 2010; Craven-Bartle *et al*, 2013; Chezem *et al*, 2016; Nemesio-Gorriz *et al*, 2017). MYBTFs were found in all comparisons analyzed (Table S2), e.g. PITA_000045249 MYB1R1 down regulated in C5d*x*Et5d; PITA_000058884 MYB 86 highly up regulated in C15d*x*Et15d; PITA_000078762 MYB-related 308-like up regulated in C15d*x*Et15d; PITA_000078623 tannin-related R2R3 MYB transcription partial and PITA_000078762 MYB-related 308-like both down regulated in C5d*x*C15d; PITA_000035495 MYB-like transcription factor ETC3 isoform X1 down regulated in Et5d*x*Et15d.

In gymnosperms, MYBTFs are known to be involved in seed development, germination, control of differentiation, vascular organization, phenylpropanoid and lignin biosynthesis, suggesting they form an ancient central transcriptional regulatory component of conifer gene networks (Bedon *et al.*, 2007; Craven-Bartle *et al.*, 2013; Chezem and Clay, 2016; Nemesio-Gorriz *et al.*, 2017). Besides lignin accumulation, MYBTFs are linked to flavonoid production, ammonium assimilation, and several of them have been observed in *Picea glauca* and *Pinus taeda* exposed to stresses such as wounding, jasmonic acid treatment, or low temperatures (Bedon *et al.*, 2010). In conifers

Pt/PgMYB14 is possibly involved in regulation of an isoprenoid-guided response that leads to accumulation of sesquiterpene (Bedon *et al.*, 2010).

A gene with reduced expression in the C5d*x*Et5d comparison encodes for an isoeugenol synthase (PITA_000091662; Table S2). Isoeugenol synthase belongs to a class of phenylpropanoid reductases, which have been shown to participate in the biosynthesis of constitutive and induced defense-related phenylpropanoids and phytoalexins, such as lignans and isoflavans, with homologs described in loblolly pine and white spruce (Porth *et al.*, 2011). The expression profile of this gene may be due to a greater effort in synthesis of other defense components, such as terpenes for resin production, in detriment of phenolic compounds (Aloni, 2013).

In the comparison C5d*x*Et5d an increase in chloroplast phenylalanine hydroxylase (PITA_000021714) expression was observed (Table S2). The enzyme encoded by this gene is involved in aromatic amino acid metabolism (Pribat *et al.*, 2010). The protein acts as a link between the phenylalanine and tyrosine branches of aromatic metabolism, which lead to different sets of products, such as benzoates, soluble phenylpropanoids and, in land plants, lignin (in Phe branch) or plastoquinones, tocopherols and degradation of the aromatic ring (Tyr branch). In gymnosperms, it has been estimated that at least under certain circumstances approximately 30% of the carbon fixed in photosynthesis is diverted to the synthesis of lignin via the Phe branch (Pribat *et al.*, 2010). Synthesis of enzymes related to Phe metabolism in trees undergoing resinosis may be involved in response to stress and cell wall regeneration, particularly in presence of ethylene.

4.4 Cell wall restoration

In C5d*x*C15d, an increase in pectinesterase (PITA_000066929) expression is observed (Table S2). The enzymes of pectin methylesterase (PME) family catalyze the demethylesterification of pectinaceous polysaccharides, which have strong influence on the colloidal water-holding capacity of pectin gels (Willats *et al.*, 2001). In *Populus*, PMEs are a gene family with several biological roles, including wood formation, vegetative and reproductive processes, being localized in expanding wood cells with an effect on cell/fiber width and length (Siedlecka *et al.*, 2008). In *Eucalyptus pilularis*, PMEs are associated with cellulose, pulp yield characteristics, being inversely related to lignin (Sexton *et al.*, 2012). In grapevine some PMEs probably participate in the regulation of fruit development and defense against pathogen invasion (Xie and Wang, 2016). In transgenic aspen PME alteration promotes oxidative stress, which along with jasmonate and ethylene signaling, is a key element in induction of tyloses (parenchyma cells ingrowth into embolized vessels that protect against pathogens) (Lesniewska *et al.*, 2017).

Expansins were also differentially expressed in some comparisons. These genes encode cell wall proteins involved in expansion and relaxation in a pH dependent mode (Mateluna *et al.*, 2017). The following expansin genes were differentially expressed: expansin B3-like (PITA_000032007) down regulated in C5d*x*C15d and in C5d*x*Et5d; and another expansin B3-like (PITA_000032006) up regulated in C5d*x*C15d (Table S2). Expansins participate in xylem secondary wall formation processes and can be influenced by factors such as water stress and phytohormones, including ABA, IAA, brassinosteroids, cytokinins and ethylene (Marowa *et al.*, 2016). In *Eucalyptus globulus* the

expression of expansins is associated with increased cellulose and hemicellulose production, as well as cell wall modification to accommodate newly formed polysaccharides (Salazar *et al.*, 2013). The presence of this DEG in our data likely reflects a response after ethylene-induced damage during resination, as well as recovery and regeneration of the cell wall of injured tissues.

The overall higher expression of oxidative stress-related genes in C5d*x*Et5d (Fig. 3a and Fig. S2a) may reflect the wound-prolonging activity of the stimulant paste. Under the same conditions and comparison, the observed higher expression of cell morphology-related genes is probably associated with resin duct differentiation mediated by ethylene. In fact, increased participation of genes related to cell wall and structural cellular components was also observed in C5d*x*Et5d and C15d*x*Et15d, which is required for resin duct development (Fig. 3a and Fig. S2a).

Chitinase presented a contrasting profile in the present assays, since its expression was repressed in C5d*x*Et5d (PITA_000006240) but induced in Et5d*x*Et15d (Table S2). Chitinases are enzymes that hydrolyze the N-acetylglucosamine polymer chitin, which may be expressed constitutively basal levels, but strongly enhanced by abiotic and biotic factors (Punja and Zhang, 1993). There is evidence that chitinases can mediate developmental processes in white spruce, acting from active growth to dormancy, playing putative roles in defense, nitrogen storage, and cell maturation prior to growth arrest (González *et al.*, 2015). The observed profile is consistent, since ethylene plays a major role in regulating plant defense responses (Khan *et al.*, 2017).

In the comparison C5d*x*Et5d an increase in expression of LP3 (PITA_000006868) inducible water deficit gene was detected (Table S2). LP3 is a water-deficit-induced protein, which increases in response to polyethylene glycol, ABA, methyl-jasmonate, and fluridone, but not by GA, 2-methyl-4-dichlorophenoxy acetic acid (2,4-D), silver nitrate, or ethylene treatments when heterologously expressed in tobacco leaves (Wang *et al.*, 2003). In *P. taeda* LP3 is preferentially induced in roots with a constitutive basal level of expression also recorded in stems and needles, but showing down regulation in stems under severe water deficit (Padmanabhan *et al.*, 1997). Differential expression of *LP3* gene family has been associated with drought in *P. taeda*, cold adaptation in *Pinus sylvestris* and xylem development in *Pinus pinaster*. In *P. taeda* significant associations between *LP3* and latewood density have been detected, suggesting their involvement in pine growth (Cabezas *et al.*, 2015). Multiple chitinase and LP3 proteins were induced by water deficit, indicating involvement of these genes in loblolly pine drought response. Monoterpenes, sesquiterpenoids, and diterpenoids, in particular, resin acids contents are positively correlated with moderate water deficit in *Pinus sylvestris* and *Picea abies*, which concurs with observations that synthesis and composition of the major components of resin is altered in wood and needle under water restriction conditions (Sancho-Knapik *et al.*, 2017). An elevated amount of resin can be observed under water-limited growth conditions, and its function may be related to the preformed defense system serving to protect against attacks or diseases that are more likely to occur on trees weakened by previous stresses and/or with wood formation changes caused by these stresses (Lautner, 2013).

4.5 UDP-sugar transferases

Several genes annotated as UDP-sugar transferases were found in all comparisons, with distinct expression patterns (C5d*x*Et5d: down regulated PITA_000014566 UDP-rhamnose:rhamnosyltransferase 1 and PITA_000057878 UDP-glycosyltransferase 74G1-like, up regulated PITA_000003789 UDP-glycosyltransferase 72B1-like; C15d*x*Et15d: up regulated 5A_I16_VO_L_4_T_65838/214906|m.48071 UDP-glycosyltransferase 86A1-like and PITA_000037586 UDP rhamnose: rhamnosyltransferase 1; C5d*x*C15d: down regulated PITA_000037271 UDP-glycosyltransferase 74E2-like and PITA_000042519 UDP-rhamnose: rhamnosyltransferase 1; Et5d*x*Et15d: down regulated PITA_000023761 UDP-glycosyltransferase 86A1-like)(Table S2). Although UDP-sugar transferases are better described in animals, recent data suggest that they may act as an extracellular signaling molecule in plants, with the possibility of being perceived as a damage-associated molecular pattern (Rensburg and Ende, 2018). Glycosylation via UDP-transferases enables the syntheses of a greater diversity of molecules with different complexities and metabolic activities (de Costa *et al.*, 2017). All major classes of secondary metabolites, such as phenolics, terpenoids, cyanohydrins, thiohydroximates (glucosinolate precursors) and alkaloids, are sugar acceptors (Vogt and Jones, 2000). These transferases can contribute to the regulation of phenylpropanoid localization, concentration and bioactivity, thereby potentially affecting resistance of plants during infection by microorganisms and adaptive responses to environmental changes (Le Roy *et al.*, 2016).

5. Conclusions

Overall, the most prominent transcriptomic responses identified during slash pine resinosis involved phytohormone-responsive genes (mainly to ethylene, jasmonate and auxin), cell wall restoration, lignification, resin duct formation, and response to oxidative stress. All of these processes are consistent with defense response to bark wounding in conifers, encompassing integrated biochemical and structural changes, particularly related to terpene production and tissue regeneration. These differences in the expression profile were observed both as a function of adjuvant presence in resin stimulating paste and in relation to resinosis time course.

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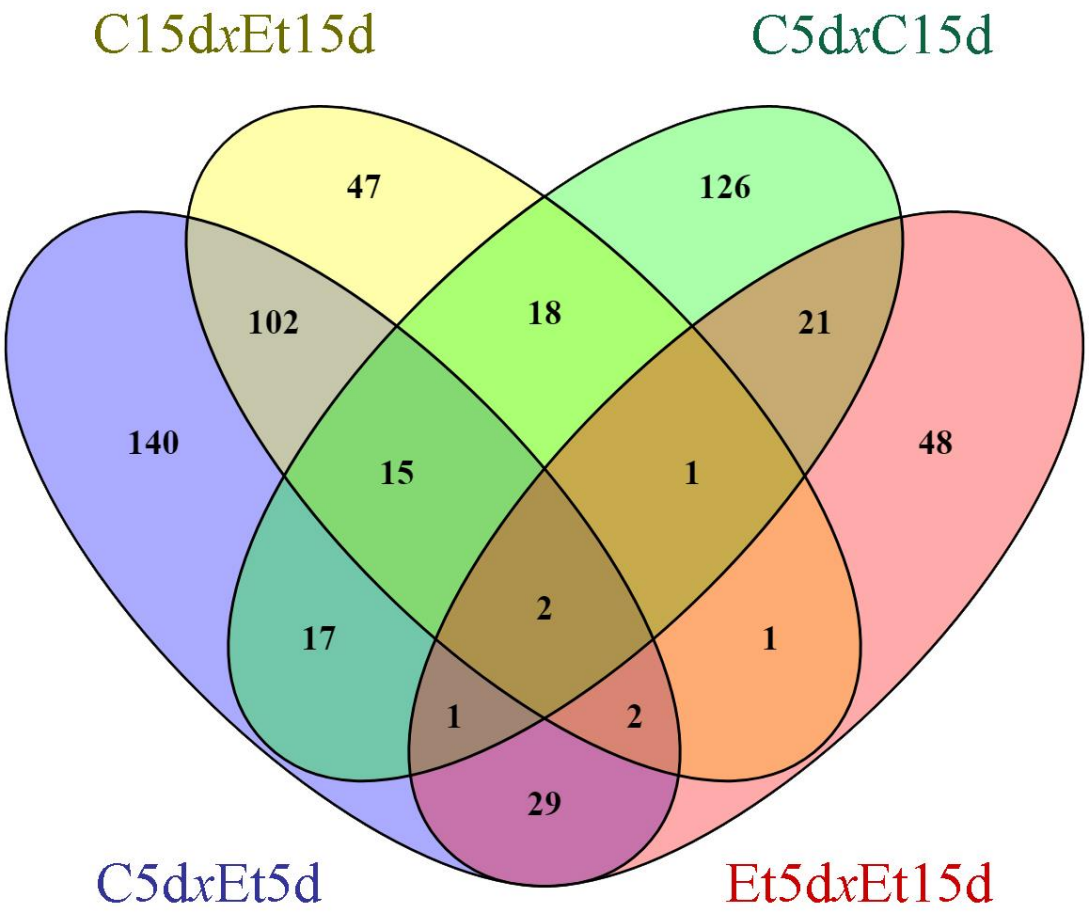
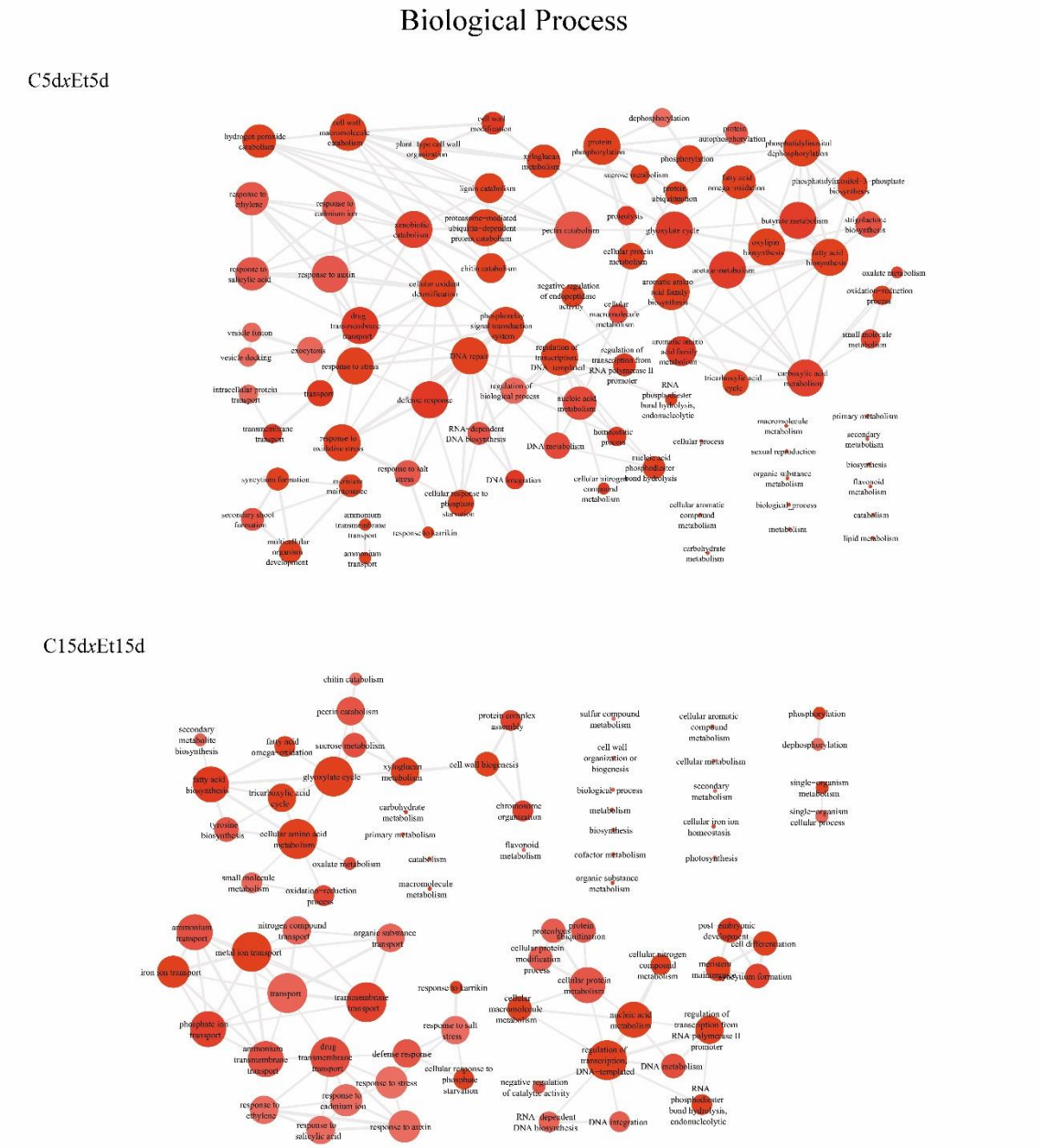
Supplementary Figures from "Resin tapping transcriptome in adult slash pine (*Pinus elliottii* var. *elliottii*)"

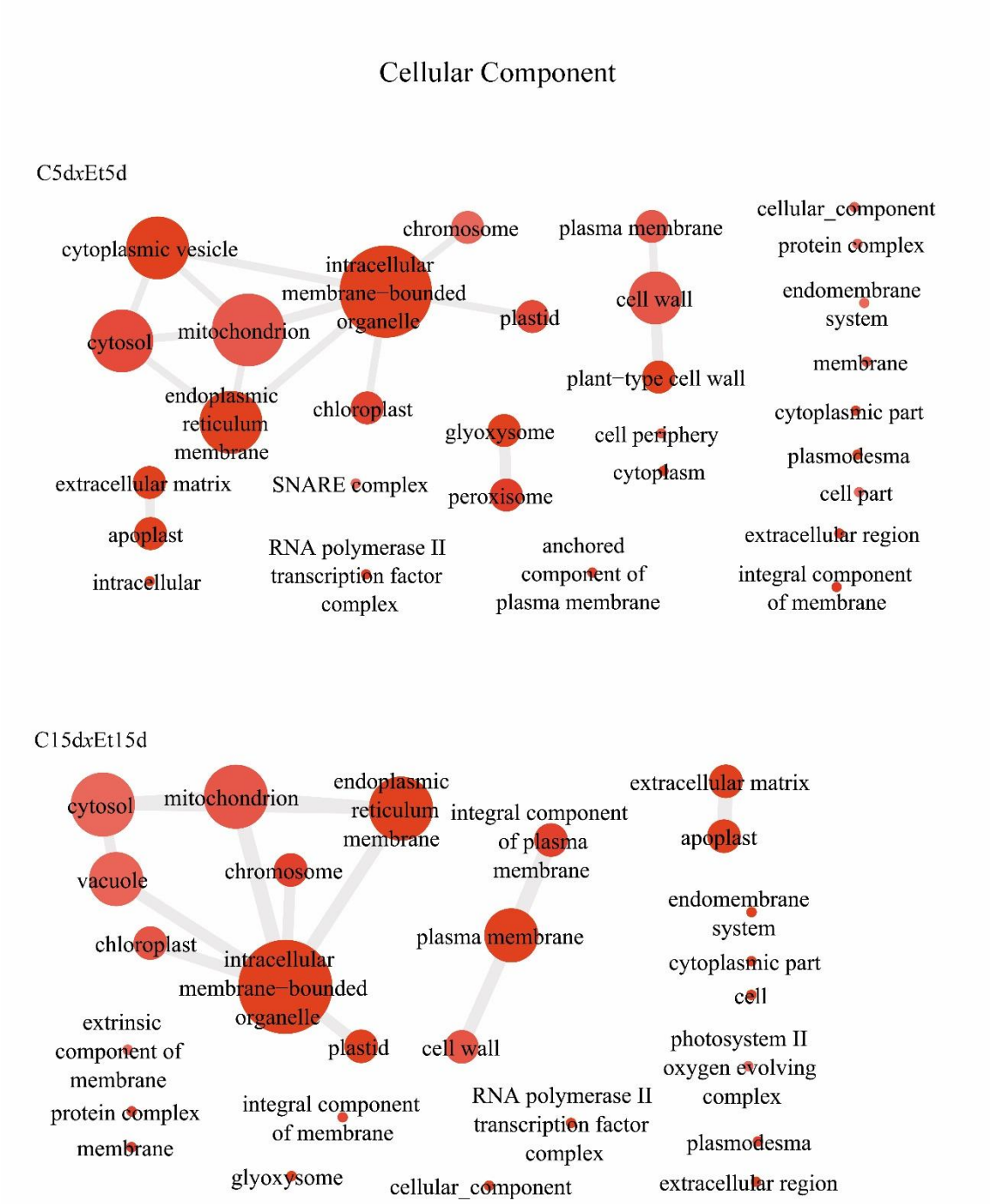


Supplementary Figure 1. Chart of common DEGs in transcriptomic analysis. Venn diagram showing distribution of DEGs in the comparisons C5d*x*Et5d, C15d*x*Et15d, C5d*x*C15d and Et5d*x*Et15d.

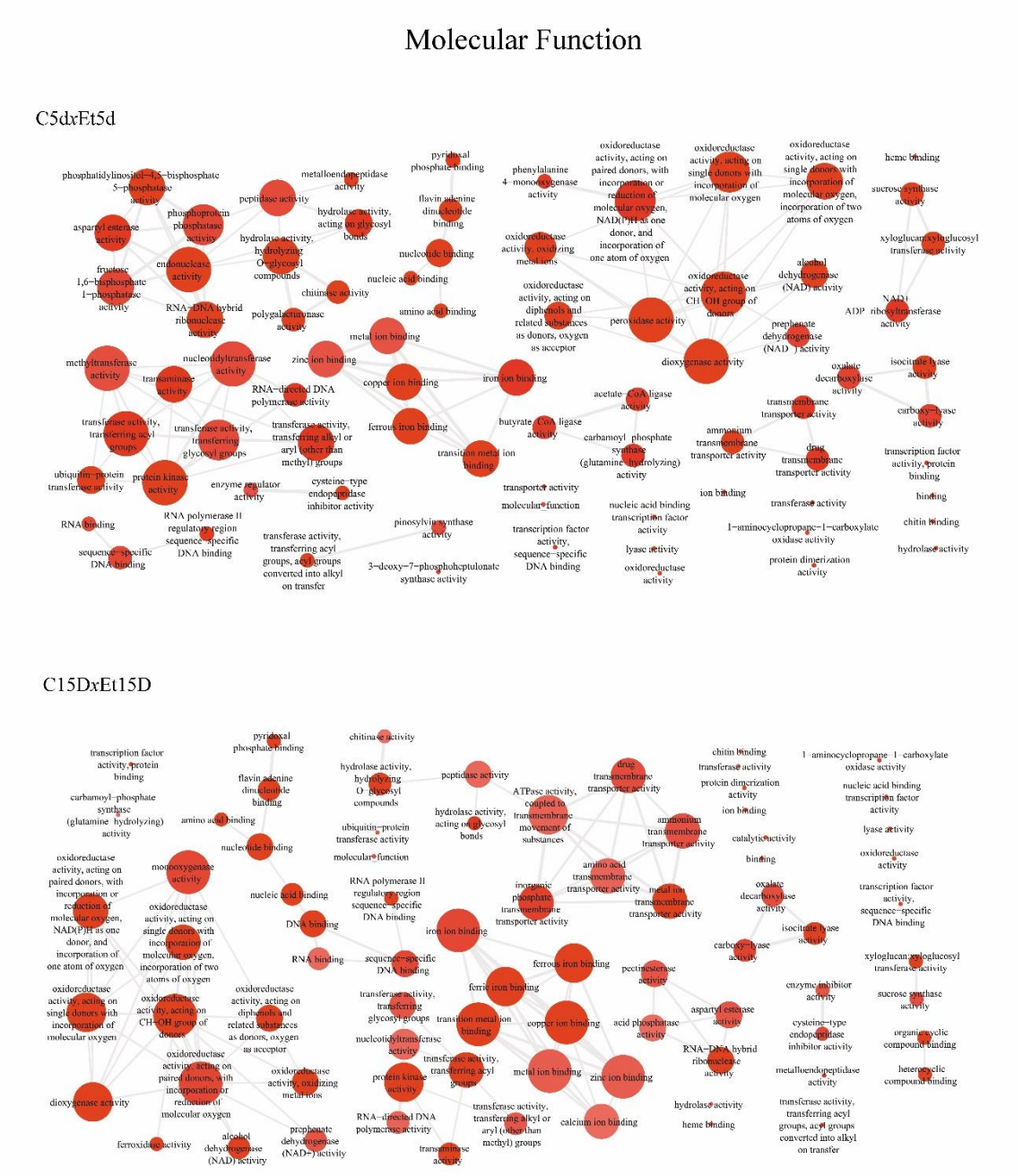
A)


Biological Process
C5dxEt5d
hydrogen peroxide catabolism
cell wall macromolecule catabolism
cell wall modification
plant-type cell wall organization
dephosphorylation
protein autophosphorylation
protein phosphorylation
phosphatidylinositol dephosphorylation
xyloglucan metabolism
phosphorylation
sucrose metabolism
fatty acid omega-oxidation
phosphatidylinositol phosphate biosynthesis
response to ethylene
response to cadmium ion
lignin catabolism
protein ubiquitination
xenobiotic catabolism
proteasome-mediated ubiquitin-dependent protein catabolism
pectin catabolism
proteolysis
glyoxylate cycle
butyrate metabolism
sesquiterpene biosynthesis
response to salicylic acid
response to auxin
chitin catabolism
cellular protein metabolism
oxylipin biosynthesis
fatty acid biosynthesis
cellular oxidant detoxification
arginine metabolism
oxalate metabolism
negative regulation of endopeptidase activity
aromatic amino acid family biosynthesis
oxidation-reduction process
drug transmembrane transport
phospholipid signal transduction system
small molecule metabolism
vesicle fusion
exocytosis
vesicle docking
response to stress
DNA repair
regulation of transcription, DNA-templated
regulation of transcription from RNA polymerase II promoter
aromatic amino acid family metabolism
intracellular protein transport
transport
defense response
regulation of biological process
nucleic acid metabolism
RNA phosphodiester bond hydrolysis, endonucleolytic
tricarboxylic acid cycle
carboxylic acid metabolism
primary metabolism
transmembrane transport
response to oxidative stress
RNA-dependent DNA biosynthesis
DNA metabolism
homeostatic process
cellular process
macromolecule metabolism
secondary metabolism
sexual reproduction
response to salt stress
nucleic acid phosphodiester bond hydrolysis
biosynthesis
syncytium formation
meristem maintenance
DNA replication
cellular nitrogen compound metabolism
organic substance metabolism
flavonoid metabolism
cellular response to phosphate starvation
cellular aromatic compound metabolism
biological process
catabolism
secondary shoot formation
ammonium transmembrane transport
response to karrikin
metabolism
multicellular organism development
ammonium transport
carbohydrate metabolism
lipid metabolism
C15dxEt15d
chitin catabolism
pectin catabolism
protein complex assembly
sulfur compound metabolism
cellular aromatic compound metabolism
phosphorylation
secondary metabolite biosynthesis
fatty acid omega-oxidation
sucrose metabolism
cell wall biogenesis
cell wall organization or biogenesis
dephosphorylation
fatty acid biosynthesis
glyoxylate cycle
xyloglucan metabolism
cellular metabolism
single-organism metabolism
tricarboxylic acid cycle
biological process
secondary metabolism
tyrosine biosynthesis
carbohydrate metabolism
chromosome organization
metabolism
single-organism cellular process
cellular amino acid metabolism
primary metabolism
biosynthesis
cellular iron ion homeostasis
flavonoid metabolism
cofactor metabolism
photosynthesis
oxalate metabolism
catabolism
small molecule metabolism
oxidation-reduction process
macromolecule metabolism
organic substance metabolism
ammonium transport
nitrogen compound transport
organic substance transport
protein folding
post-embryonic development
metal ion transport
protein lipidation
cellular protein modification process
cell differentiation
cellular nitrogen compound metabolism
iron ion transport
transport
transmembrane transport
response to karrikin
cellular protein metabolism
meristem maintenance
syncytium formation
cellular macromolecule metabolism
nucleic acid metabolism
phosphate ion transport
response to salt stress
regulation of transcription from RNA polymerase II promoter
ammonium transmembrane transport
drug transmembrane transport
defense response
regulation of transcription, DNA-templated
DNA metabolism
cellular response to phosphate starvation
response to stress
negative regulation of catalytic activity
response to cadmium ion
RNA phosphodiester bond hydrolysis, endonucleolytic
response to ethylene
response to auxin
RNA-dependent DNA biosynthesis
DNA integration
response to salicylic acid

B)

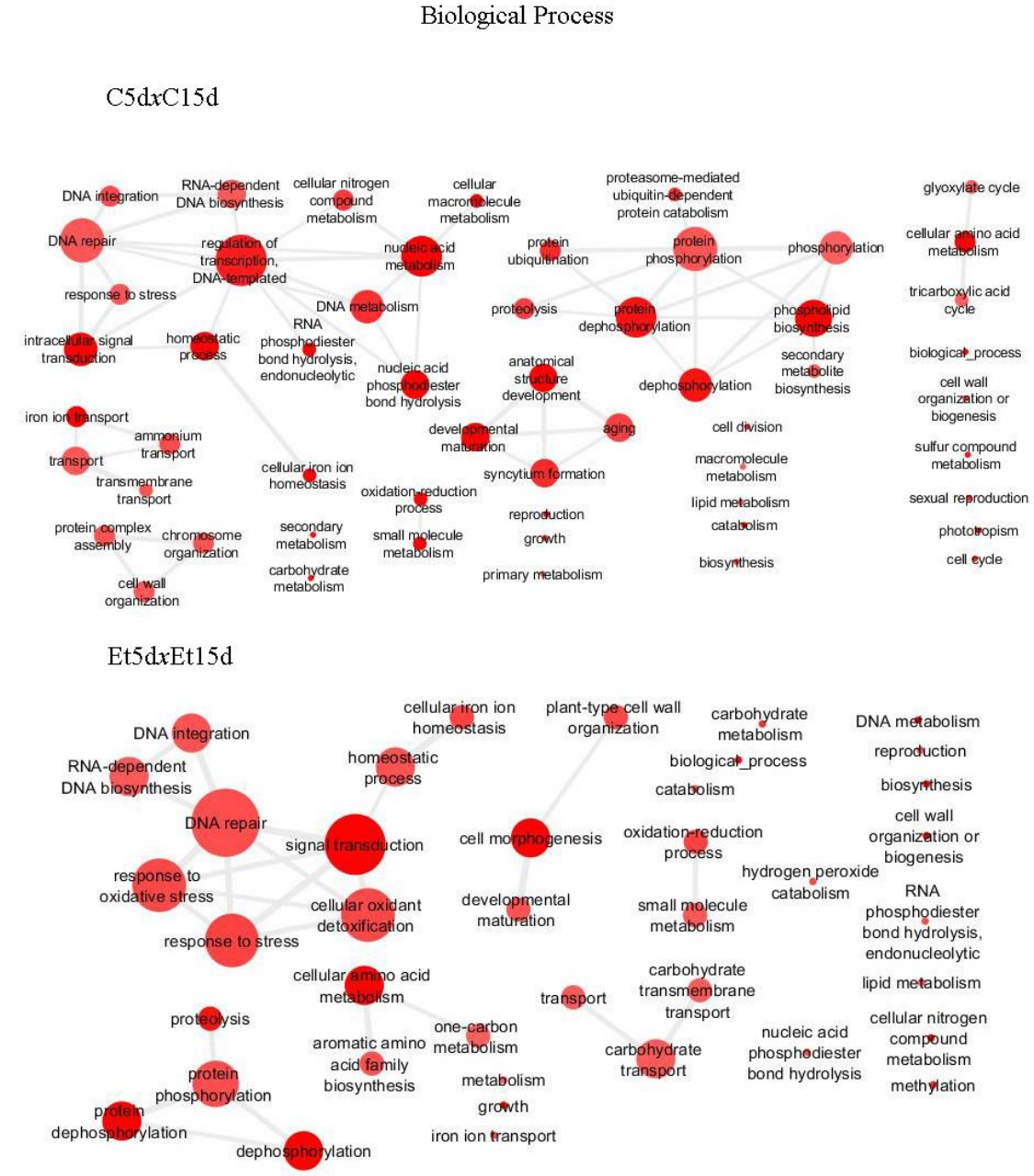

Cellular Component
C5dxEt5d
cytoplasmic vesicle
chromosome
plasma membrane
cellular_component
protein complex
intracellular membrane−bounded organelle
cell wall
endomembrane system
cytosol
mitochondrion
plastid
membrane
plant−type cell wall
endoplasmic reticulum membrane
chloroplast
glyoxysome
cytoplasmic part
cell periphery
plasmodesma
extracellular matrix
SNARE complex
cytoplasm
peroxisome
cell part
apoplast
extracellular region
RNA polymerase II transcription factor complex
anchored component of plasma membrane
integral component of membrane
intracellular
C15dxEt15d
cytosol
mitochondrion
endoplasmic reticulum membrane
integral component of plasma membrane
extracellular matrix
apoplast
vacuole
chromosome
endomembrane system
plasma membrane
chloroplast
intracellular membrane−bounded organelle
cytoplasmic part
cell
extrinsic component of membrane
plastid
cell wall
photosystem II oxygen evolving complex
protein complex
integral component of membrane
RNA polymerase II transcription factor complex
membrane
plasmodesma
glyoxysome
cellular_component
extracellular region

C)

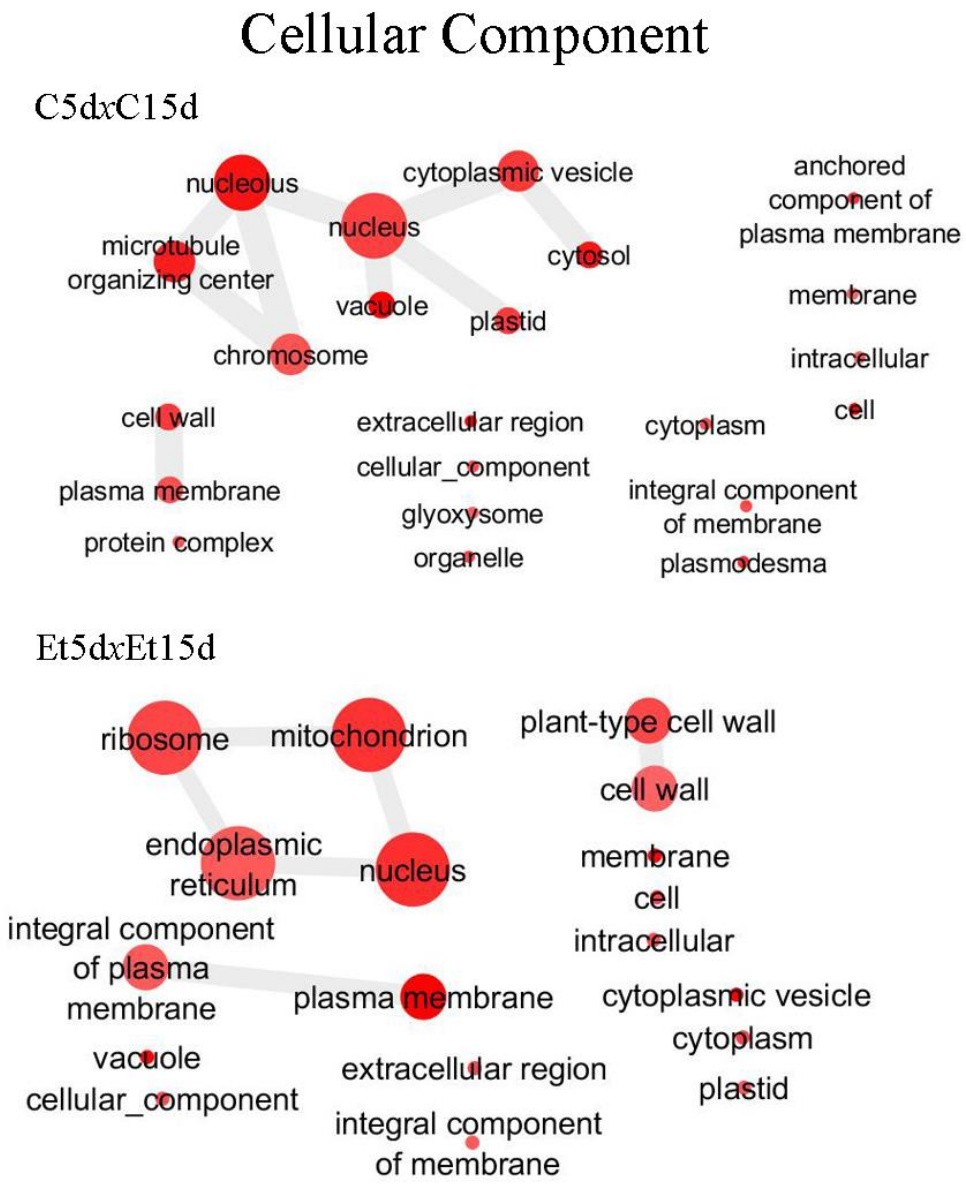


Supplementary Figure 2. GO enrichment analysis of DEGs between stimulant paste with Ethrel or control effect on oleoresin-yielding slash pines using REVIGO. **A)** Biological processes, **B)** Cellular component and **C)** Molecular function interactive graph of the enriched GO terms for DEGs on C5d*x*Et5d (top of the chart) or C15d*x*Et15d (bottom of the chart) comparisons. Bubble color intensity indicates the p-value; bubble size represents the frequency of the GO term (more general terms are represented by larger size bubbles). Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.

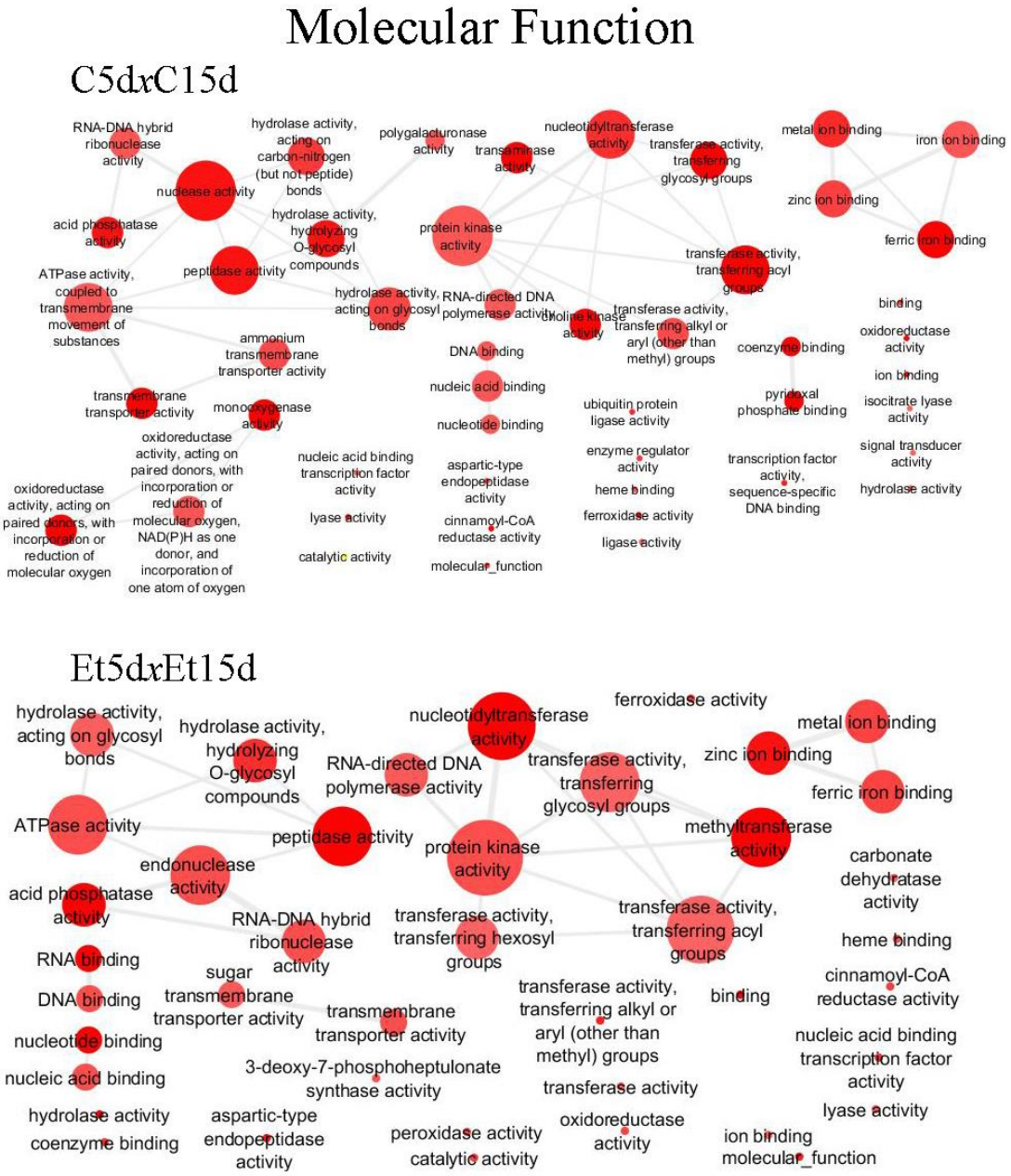
A)


Biological Process
C5dxC15d
DNA integration
RNA-dependent DNA biosynthesis
cellular nitrogen compound metabolism
cellular macromolecule metabolism
proteasome-mediated ubiquitin-dependent protein catabolism
glyoxylate cycle
DNA repair
regulation of transcription, DNA-templated
nucleic acid metabolism
protein ubiquitination
protein phosphorylation
phosphorylation
cellular amino acid metabolism
response to stress
DNA metabolism
proteolysis
protein dephosphorylation
phospholipid biosynthesis
tricarboxylic acid cycle
intracellular signal transduction
homeostatic process
RNA phosphodiester bond hydrolysis, endonucleolytic
nucleic acid phosphodiester bond hydrolysis
anatomical structure development
dephosphorylation
secondary metabolite biosynthesis
biological_process
cell wall organization or biogenesis
iron ion transport
ammonium transport
transport
transmembrane transport
developmental maturation
aging
cell division
sulfur compound metabolism
cellular iron ion homeostasis
syncytium formation
macromolecule metabolism
oxidation-reduction process
lipid metabolism
sexual reproduction
protein complex assembly
chromosome organization
secondary metabolism
small molecule metabolism
reproduction
growth
catabolism
phototropism
cell cycle
carbohydrate metabolism
primary metabolism
biosynthesis
cell wall organization
Et5dxEt15d
cellular iron ion homeostasis
plant-type cell wall organization
carbohydrate metabolism
DNA metabolism
DNA integration
homeostatic process
biological_process
reproduction
RNA-dependent DNA biosynthesis
catabolism
biosynthesis
DNA repair
signal transduction
cell morphogenesis
oxidation-reduction process
cell wall organization or biogenesis
response to oxidative stress
hydrogen peroxide catabolism
cellular oxidant detoxification
developmental maturation
small molecule metabolism
RNA phosphodiester bond hydrolysis, endonucleolytic
response to stress
cellular amino acid metabolism
carbohydrate transmembrane transport
lipid metabolism
transport
proteolysis
one-carbon metabolism
nucleic acid phosphodiester bond hydrolysis
cellular nitrogen compound metabolism
aromatic amino acid family biosynthesis
carbohydrate transport
protein phosphorylation
metabolism
methylation
protein dephosphorylation
growth
dephosphorylation
iron ion transport

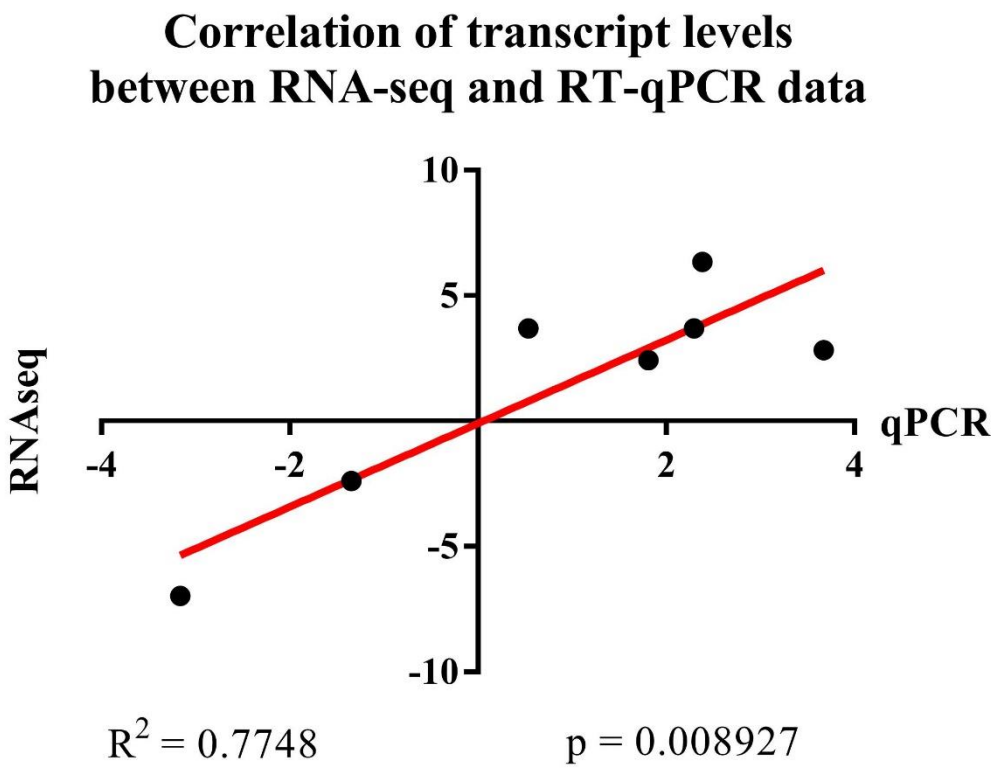
B)


Cellular Component
C5dxC15d
nucleolus
cytoplasmic vesicle
anchored component of plasma membrane
nucleus
microtubule organizing center
cytosol
membrane
vacuole
plastid
chromosome
intracellular
cell wall
extracellular region
cytoplasm
cell
cellular_component
plasma membrane
integral component of membrane
glyoxysome
protein complex
organelle
plasmodesma
Et5dxEt15d
ribosome
mitochondrion
plant-type cell wall
cell wall
endoplasmic reticulum
nucleus
membrane
cell
integral component of plasma membrane
intracellular
plasma membrane
cytoplasmic vesicle
cytoplasm
vacuole
extracellular region
plastid
cellular_component
integral component of membrane

C)



Supplementary Figure 3. GO enrichment analysis of DEGs between five or fifteen days of treatment effect on oleoresin-yielding slash pines using REVIGO. **A)** Biological processes, **B)** Cellular component and **C)** Molecular function interactive graph of the enriched GO terms for DEGs on C5d*x*C15d (top of the chart) or Et5d*x*Et15d (bottom of the chart) comparisons. Bubble color intensity indicates the p-value; bubble size represents the frequency of the GO term (more general terms are represented by larger size bubbles). Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.



Supplementary Figure 4. Validation of RNA-seq results by quantitative real time PCR. Correlation plots indicating the relationship between RT-qPCR results (log_2(FC); x-axis) of selected genes expressed in the cambium of slash pine on resinosis and the corresponding data from RNA-seq analysis (log_2(FC); y-axis). Data show agreement according to Pearson's correlation.

VII. Considerações Finais e Perspectivas

A utilização de resina de coníferas é uma prática milenar da humanidade. Considerando a versatilidade de produtos obtidos a partir deste material e a necessidade de encontrar novas fontes renováveis de biopolímeros para a indústria, esforços vêm sendo dedicados para aumentar a produção de resina por árvore, sem que seja necessário expandir demasiadamente áreas de plantio.

Estudos já mostraram que a produção comercial tem significativo aumento quando são realizados estriamentos periódicos [geralmente em intervalos de 15 dias], seguidos da aplicação de pastas adjuvantes sobre o dano. Além disso, pastas contendo precursores sintéticos de etileno [Ethrel], de ácido salicílico [ácido benzoico], ou sulfato de potássio resultam em ganhos consideráveis de produção a campo.

Este trabalho tratou sobre a questão de resinagem de *Pinus elliottii* em duas frentes principais: a primeira visando avaliar a utilização de plantas jovens como sistema para prospecção de pastas com potencial de indução de resinagem, método que denominamos “microrresinagem”. Para isso, foram utilizados compostos com conhecido potencial de indução a campo, além de metil-jasmonato como controle positivo. Em plantas de 1, 2 ou 3 anos de idade percebemos uma resposta similar à observada em plantas adultas quando à capacidade de indução periódica da produção de resina. Foi observado que pastas com maior potencial de indução resultam em diferenças significativas para a maior produção de resina, principalmente em pontos basais do caule.

Embora alguns compostos com capacidade indutora já estabelecida a campo não tenham sido passíveis de diferenciação estatística no estímulo à resinose em experimentos de microrresinagem, os controles positivos de metil jasmonato e Ethrel apresentaram impacto significativo em plantas de 2 e 3 anos crescidas em casa de vegetação. Isso nos leva a crer que este sistema pode ser útil na identificação de adjuvantes com alta capacidade de indução, em condições controladas de laboratório/casa de vegetação e em menos tempo do que seria necessário para validar estes compostos a campo. Desta forma, um maior conjunto de moléculas e combinações de compostos pode ser testado de forma mais simples, rápida e com menor custo de implantação e mão-de-obra.

Uma consequência inesperada destes estudos de microrresinagem foi a identificação de indivíduos com perfis contrastantes de produção de resina após a análise dos dados do primeiro ano. Aos três anos, estes indivíduos, denominados “muito produtores de resina” ou “pouco produtores de resina” foram novamente avaliados quanto à produção de resina, confirmando seu fenótipo inicial. Além disso, a expressão de genes codificadores de algumas enzimas e fatores de transcrição relacionados à síntese de terpenos também foi diferenciada em indivíduos com perfil de maior produção de resina. O perfil observado nos mostra que o padrão de expressão para α- e β-pineno sintase está de acordo com o esperado para a quantificação dos metabólitos resultantes em plantas adultas pouco ou superresinosas. Além disso, plantas jovens classificadas como muito produtoras de resina apresentaram maior expressão de um fator de transcrição de resposta a etileno, que também foi identificado em análises transcriptômicas de resinagem em plantas adultas de *P. elliottii*. Estes fatos indicam que estas plantas apresentam desde o início do seu desenvolvimento uma maior capacidade de perceber e responder ao dano mecânico comparativamente a plantas pouco produtivas de resina.

Novos experimentos serão conduzidos com estas plantas, especificamente análises de expressão de outros genes que possam estar envolvidos na seleção de indivíduos com alta capacidade de resinose, como transportadores do tipo ABC, por exemplo. Além disso, o perfil de expressão de diversos genes destas plantas será comparado com o observado em plantas adultas pouco e superresinosas, a fim de que possam ser constatadas as similaridades existentes. Por fim, estas plantas serão mantidas até a idade adulta, para verificar se os respectivos fenótipos serão mantidos. Os dados obtidos, no entanto, indicam que o método de microrresinagem representa uma ferramenta também útil para a seleção precoce de indivíduos elite, permitindo, de maneira mais eficiente e rápida, que se estabeleçam florestas com maior ganho de produção de resina por planta. Além disso, esta técnica pode proporcionar um maior sucesso na propagação clonal de plantas com fenótipo de interesse, já que este processo é facilitado quando plantas jovens são utilizadas. Por outro lado, a identificação de marcadores moleculares funcionais definidos para resinagem poderá servir para programas de melhoramento com foco na maior produção de resina desta importante espécie florestal.

A segunda frente abordada por esta tese teve como foco principal descrever o perfil transcriptômico durante o processo comercial de resinagem via RNAseq. Para isso, foram utilizadas plantas adultas que já vinham sendo resinadas comercialmente por dois anos. Os ferimentos foram tratados sem pasta [controle] ou com pasta contendo Ethrel. Cinco ou quinze dias após o dano, amostras do lenho jovem foram coletadas logo acima do painel de resinagem; a partir destas amostras foram obtidos os dados de expressão gênica. Nossos resultados mostraram uma intrincada e complexa rede de regulação gênica, envolvida tanto no metabolismo primário como secundário para todas as comparações, sejam em relação ao efeito do tempo de indução ou à exposição ao tratamento com pasta adjuvante. Diversos genes relacionados a estresse oxidativo e à restauração de parede celular foram diferencialmente expressos, além de genes relacionados à ação e metabolismos de fitormônios, como auxina, etileno e giberelinas. Outros genes incluíram fatores de transcrição do tipo MYB; genes responsivos a jasmonato ou ligados a mecanismos de defesa, como lipoxigenases, terpeno sintases, CYP450s, quitinases, etc. O perfil de expressão de alguns destes genes foi comparado por RT-qPCR, havendo consistência entre os dados de RNAseq e RT-qPCR. Estes dados fornecerão uma base bastante valiosa para estudos envolvendo a biologia molecular da indução de resina, com identificação de possíveis alvos na prospecção de novos indutores, marcadores de resinose, ou novas metodologias de produção. A identificação de transcritos de *P. elliottii* via RNAseq também será útil para diversas pesquisas, visto que esta espécie ainda não teve o seu genoma sequenciado.

Os resultados obtidos nesta tese ressaltam a importância do uso de abordagens diversas e complementares na busca de soluções para problemas encontrados na indústria florestal. Tanto metodologias simples, como a utilização de plantas jovens para facilitar a seleção de pastas indutoras e de indivíduos de maior potencial de produção com redução de custo, tempo e trabalho, quanto abordagens relativamente mais complexas, como análises transcriptômicas para o melhoramento genético e do manejo de plantações, podem ajudar a resolver gargalos da produção de resina de pinus.

VIII. Anexo 1

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

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Keywords:
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microCT

ABSTRACT

In conifer stems, secretory canals synthesize and store resin for defense against herbivores and pathogens. Resin terpenes are used as raw material by an array of industrial sectors. Most forest stands operationally used in resin extraction are derived from seeds, showing high genetic variation, which reflects in yield. The objective of this study was to identify adult slash pine (*Pinus elliottii* Engelm.) trees of high yield of resin in a short timeframe by resin mass flow rate analysis, aiming at the establishment of elite forests for 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. High yielding trees had more axial resin canals when compared with low yielding ones. Frequency of putative 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 lower in more productive trees, which also had more limonene in total terpenes compared with their low yield counterparts. Data support the use of short-term resin mass flow rate analysis as a tool to identify and select high yield trees for the establishment of elite slash pine forests for resin tapping operations. Strong correlation of the superesinous phenotype with canal density and structure was also evident.

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 canals), 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 commonly induced response to mechanical damage, insect attack, fungal invasion, application of hormones and chemical stimulants is the production of traumatic resin canals in the xylem (Lombardero et al., 2000; Nagy et al., 2000; Franceschi et al., 2002; Arbellay et al., 2014). The formation of traumatic resin canals 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, 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). Selection of high resin yield

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IX. Anexo 2

frontiers in Plant Science

ORIGINAL RESEARCH
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Events Associated with Early Age-Related Decline in Adventitious Rooting Competence of *Eucalyptus globulus* Labill

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The development of adventitious roots is affected by several factors, including the age of the cutting donor plant, which negatively affects rooting capacity. *Eucalyptus globulus* quickly loses rooting capacity of cuttings as the donor plant ages, although the molecular and biochemical mechanisms behind this process are still unclear. To better understand the bases of rooting competence loss in *E. globulus*, the time required for a significant decline in rhizogenic ability without exogenous auxin was determined in microcuttings derived from donor plants of different ages after sowing. Tip cuttings of donor plants were severed before and after loss of rooting competence of microcuttings to test the hypothesis that auxin and carbohydrate homeostasis regulate rooting competence decline. There were no significant changes in concentration of carbohydrates, flavonoids, or proteins before and after the loss of rooting capacity. Peroxidase (EC 1.11.1.7) total activity increased with loss of rooting competence. Auxin concentration showed the opposite pattern. In good agreement, *TAA1*, a key gene in auxin biosynthesis, had lower expression after loss of rooting capacity. The same applied to the auxin receptor gene *TIR1*, suggesting reduced auxin sensitivity. On the other hand, genes associated with auxin response repression (*TPL*, *IAA12*) or with the action of cytokinins, the rhizogenesis inhibitor-related *ARR1*, showed higher expression in plants with lower rooting competence. Taken together, data suggest that age negatively affects *E. globulus* rooting by a combination of factors. Decreased endogenous auxin concentration, possibly caused by less biosynthesis, lower auxin sensitivity, higher expression of genes inhibiting auxin action, as well as of genes related to the action of cytokinins, appear to play roles in this process.

Keywords: adventitious rooting, *Eucalyptus*, juvenility, auxin, gene expression

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INTRODUCTION

Eucalyptus globulus Labill. is considered one of the top species for the paper industry due to its high quality cellulose pulp, low lignin and lipid content, and high syringyl/guaiacyl (S/G) ratio (Cruz et al., 2006; Rencoret et al., 2007; Barbosa et al., 2008; Neiva et al., 2014). However, this is a rooting recalcitrant species (Le Roux and Van Staden, 1991; Fett-Neto et al., 2001),

X. Anexo 3

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Accumulation of the antioxidant alkaloid brachycerine from *Psychotria brachyceras* Müll. Arg. is increased by heat and contributes to oxidative stress mitigation

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Oxidative stress

ABSTRACT

Brachycerine is a shoot monoterpene indole alkaloid with antioxidant, UV protectant, and antimutagenic activities present in *Psychotria brachyceras*. The alkaloid has been shown to be induced by osmotic stress, UV, heavy metals, and wounding. Since brachycerine accumulation is related to redox imbalance, herein we tested the hypothesis that heat induces accumulation of the alkaloid, which helps mitigating oxidative stress. Brachycerine concentration in leaf disks exposed to 40 °C for three days, both by abrupt and stepwise temperature raise, increased by 4.5 and 2 fold, respectively, reaching up to 2.0% of the extracted dry weight. Alkaloid concentration was not affected by exposure to 10 °C. Lipid peroxidation was reduced in *P. brachyceras* under acute and stepwise heat stress compared to control condition. Hydrogen peroxide concentration was lower in leaf disks exposed to heat shock (50 °C) compared to control. No changes were observed in total chlorophyll under any of the temperature treatments. Leaf disks of the heat-sensitive species *Brugmansia suaveolens* and *Brassica oleracea*, which had massive loss of chlorophyll under heat, showed heat shock tolerant phenotype when pre-treated with brachycerine in concentrations equivalent to those found in *P. brachyceras*. Expression of *TRYPTOPHAN DECARBOXYLASE* (*TDC*), encoding an enzyme involved in alkaloid biosynthesis, was repressed in leaf disks exposed to 40 °C for 6, 12 and 24 h, suggesting that temperature action may take place at post-transcriptional level. In fact, heat exposed-disks had higher concentration of the alkaloid precursor tryptamine and TDC activity compared to control counterparts. Taken together, data shows that accumulation of brachycerine is induced by heat, probably by a post-transcriptional mechanism, contributing to protection against associated oxidative damage.

1. Introduction

Plants are subject to fluctuations in environmental temperature, both daily and seasonal, throughout their life cycle. Heat stress is defined as the increase in temperature relative to the values considered optimal for a plant, lasting long enough to impair its growth and development (Wahid et al., 2007). The damages caused by heat stress include changes in permeability of cell membranes, denaturation and aggregation of proteins, cytoskeletal instability, and decoupling of primary metabolism by enzymatic inactivation. These damages can alter differentiation, elongation and expansion of plant cells, reduce cellular ion flow and lead to increased reactive oxygen species production (Bita and Gerats, 2013).

Brachycerine is the major monoterpene indole alkaloid (MIA) synthetized by *Psychotria brachyceras* Müll. Arg. (Rubiaceae). This alkaloid is restricted to shoots and induced in leaves by several stimuli, such as UV light, mechanical damage, jasmonic acid (JA), heavy metals, osmotic stress, and abscisic acid (ABA) (Gregianini et al., 2003, 2004; Nascimento et al., 2013a). Brachycerine has antioxidant, antimutagenic and UV protectant activity (Nascimento et al., 2007), but showed no herbivore deterrent effects in different assays (Porto et al., 2014). Brachycerine and related MIAs in *Psychotria* species can contribute to the re-establishment of metabolic redox balance in response to several environmental stresses (Matsuura et al., 2014).

Monoterpene indole alkaloids are derived from tryptophan and the iridoid terpene secologanin (Connor and Maresh, 2006). However

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XI. Anexo 4

Environmental regulation of bioactive metabolite accumulation in Brazilian medicinal plants

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ABSTRACT

Plant natural products evolved as key elements in adaptive responses to stress, both biotic and abiotic, in close connection with the sessile habit. During this process, an intricate relationship between dedicated metabolic pathways and structural features of plants was established, affording high efficiency in metabolic competence and originating great metabolite diversity. This metabolic array has proven a major reservoir of bioactive compounds for treating and preventing human diseases. Considering the defense-related role of natural products in plants and the signaling pathways that trigger their biosynthesis upon stress exposure, it may be advantageous to use environmental signals or their transduction elements for enriching biomass with pharmacologically interesting metabolites. Among the environmental factors that promote natural product accumulation when applied at moderate intensity are: heat, cold, drought, herbivory, pathogens, UV radiation, osmotic stress, and heavy metals. This chapter reviews some recent examples on stimulation of bioactive natural product accumulation in Brazilian medicinal plants, offering an integrated view of plant-environment interaction strategies to improve target metabolite yields.

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8 a 10 de agosto | Canela - RS

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MICROTAPPING OF PINUS ELLIOTTII ENGELM AS AN ALTERNATIVE IN PROSPECTING RESINOSE ADJUVANT PASTES

dos autores
JOÃO VÍTOR VIGNE DUZ, MAGNUS R. KERBER, JULIANA L. GALVAN, JÚLIA WIECZOREK, CAMILA F. O. JUNKES, ARTHUR G. FETT-NETO
obteve **Menção Honrosa** na categoria avaliação pôster no **Congresso SulBiotec 2018**, realizado de 08 a 10 de agosto de 2018, no Centro de Eventos do Hotel Encantos Canela.

Canela, 10 de agosto de 2018.

Odir Antônio Dellagostin
Coordenador SulBiotec

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Congresso SulBiotec2018
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CERTIFICADO

Certificamos que o trabalho

TRANSCRIPTOMIC ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES ASSOCIATED WITH RESINOSIS IN SLASH PINE (PINUS ELLIOTTI VAR. ELLIOTTII)

dos autores

CAMILA FERNANDA DE OLIVEIRA JUNKES, ARTUR T. ARAÚJO JR., FERNANDA DE COSTA, JANETTE P. FETT, ARTHUR G. FETT-NETO

obteve **Menção Honrosa** em primeiro lugar na categoria avaliação oral no **Congresso SulBiotec 2018**, realizado de 08 a 10 de agosto de 2018, no Centro de Eventos do Hotel Encantos Canela.

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