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Expression patterns of terpene biosynthesis-related genes and resin production analysis in seedlings of *Pinus elliottii* Engelm.

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Abstract: As sessile organisms, plants must rely on a diverse array of chemical and physiological defenses to avoid and/or tolerate abiotic and biotic stresses. Pinaceae species produce resin rich in defensive terpenes which are exploited by industry. In order to identify young plants of *Pinus elliottii* (slash pine) that produce a higher quantity of resin, three different methodologies were tested to measure this potential in plants previously known to synthesize high and low quantities of resin. Resin exuded after bark removal and application of a chemical stimulant (methyl jasmonate) was quantified in parallel with qRT-PCR evaluations of transcriptional changes of genes related to the synthesis of terpenes and ethylene-responsive transcription factors. In addition, a gravimetric analysis of the resin content in brachyblasts was performed. The first two approaches proved to faithfully reflect the seedling's capacity to yield resin, since consistent differences in resin weight and corresponding level of expression of several of the genes examined were observed for the two contrasting phenotypes. On the other hand, brachyblast resin gravimetry did not show significant difference between the phenotypes. To sum up, both resin weighing after bark removal and terpene and ethylene related gene expression properly reflected resin yield capacity, which may be useful for earlier selection of elite individuals to establish superresinous forests aiming at supplying the resin industry. In addition, resin production seems to be regulated at least partially by transcriptional activity in young slash pine trees.

1 – Introduction:

Plants face specific challenges in order to gather resources and avoid injuries because of their sessile character. Plant secondary metabolism is an important feature in plant evolution, and plays a major role in the resistance to stressful environmental factors, such as light intensity, soil salinity, drought, temperature variation and nutrient availability, as well as to biotic factors, namely herbivore attack, pathogen invasion and competition with other plants for resources (Lange, 2015; Matsuura et al., 2014).

Amongst the array of compounds produced in order to protect plants against damage, terpenes distinguish themselves for their importance and diversity, encompassing over 30,000 different chemical structures. Terpenes are components of the phytol chain in chlorophylls and precursors of gibberellins (Weiss and Ori, 2007). The ecological importance of this large class of specialized metabolites includes attraction of pollinators, predatory or parasitic insects to feed on herbivores, protection against oxidative damage caused by extreme abiotic factors, and direct defense against pathogens and herbivores (Tholl, 2006).

Terpene biosynthesis involves the assembly of C5-unit isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Prenyltransferases produce the general precursors of terpenes: geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). Terpene synthases (TPS) are enzymes that catalyze the formation of terpenes: GPP is the precursor for monoterpenes, FPP for sesquiterpenes, and GGPP for diterpenes (Tholl, 2006).

Terpenes have been used by humans since ancient times. One of the most notable sources of useful terpenes is the pine tree (*Pinus* spp.). Besides cellulose and fibers, pines produce large quantities of resin, which is stored in preformed resin ducts located in the bark, sapwood and needles (Byun-McKay et al., 2006). Resin is a blend of volatile and non-volatile terpenes, constituting a protection against herbivores and pathogens that feed on the trees (Rodrigues-Correa and Fett-Neto, 2013). Resin can be fractionated in two main portions:

turpentine, which is composed of monoterpenes and sesquiterpenes (*i.e*, molecules with two or three monomers of isoprene, respectively); and rosin, made of diterpenes (with four monomers of isoprene) (Neis et al., 2018). The main applications of this resin lie in the production of pharmaceuticals, synthetic rubber, adhesive materials, emulsifiers, paints, among others.

The most common method employed to extract resin from commercial plantations is to streak the bark with a shaving tool, in a surface corresponding roughly to one-third of the tree's circumference, in order to expose the sapwood. Usually, a resinosis stimulating adjuvant is applied on the wound, increasing terpene biosynthesis and secretion. A bag is placed beneath the cut, so as to collect the exudate (Fett-Neto and Rodrigues-Corrêa, 2012; Neis et al., 2018).

Pine trees grown in field conditions often show significant variation in resin yield. This may be due to environmental factors, which influence resinosis (Sampedro et al., 2010). However, genetic diversity among individuals is thought to be the main cause for this variation, as young plants generally are not clonal, but derived from seeds.

Plant breeding programs aiming to improve the process of identifying high resin yield individuals are often laborious, owning to the fact that the characterization of resin production per tree is made at field tapping age (around 12 to 14 years). Also, there are constraints in pine clonal propagation and a long time is needed for individuals to acquire sexual maturity. To address the problem of promoting greater resin yield, efforts are being dedicated to understand the molecular basis of adult tree resinosis (de Lima et al., 2016; Yi et al., 2018), which still remains widely unknown.

Here, we tested three different techniques to distinguish *P. elliottii* (slash pine) seedlings with higher or lower resin production potential. We compared the trees with regard to the resin extracted from micro tapping of the bark, to see whether the differences could be directly measured at early growth stages. Also, we aimed to compare expression profiles of genes involved in terpene biosynthesis in young plants of *P. elliottii* exhibiting different levels of resin production. Finally, we also conducted a gravimetric analysis of resin extracted

from brachyblasts of both phenotypes to check for a possible correlation with stem resin yield.

2. Methodology

2.1 Plant material

Previous essays using 1-year-old *Pinus elliottii* trees identified individuals with high and low resin production, selected based on their value above or below the mean standard deviation of resin yield after bark removal, *i.e.* microtapping (Junkes et al., unpublished). Selected plants were maintained in greenhouse, grown in PVC pots containing substrate + vermiculite (1:1), under natural light, and watered regularly. Average temperatures ranged between 11°C to 13°C (minimum) and 20°C to 21°C (maximum). Three methodologies were used to validate the resin phenotype in 3-year-old trees: microtapping (reference method), gene expression prolife, and gravimetric production of resin extracted from brachyblasts (short branches with needle clusters). All experiments were carried out between August and October of 2018 with 4 putative super and 4 putative low resinous plants, for all experiments.

2.2 Microtapping

The procedure of resin harvest started with a wounding procedure of approximately 1 cm on the trunk to remove the bark and expose the cambium and sapwood with aid of a scalpel. On this wound, 50 µL of an adjuvant paste consisting of aqueous and glycerol solution 1:1 (v/v) with methyl jasmonate [50 mM MeJa] was applied. After one week, resin produced by each plant was individually collected and weighed.

2.3 Gene expression

For the gene expression experiments, a portion of the bark 5 cm above and below the wound was removed and the exposed cambium and sapwood were collected for RNA extraction (1 week after MeJa application). Samples were immediately stored in liquid nitrogen after collection. Total RNA was extracted from the tissues using Concert plant RNA reagent (Life Technologies), as described by de Lima et al. (2016a). Nanodrop spectrophotometer (Thermo Scientific) was used to verify RNA concentration.

Gel electrophoresis in 1% agarose gel 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. Samples were then treated with 1 U of DNAse and 1 µL of DNAse reaction buffer (Ampgrade Invitrogen). The mixture was left for 15 minutes at room temperature. Then, 1 µL EDTA (25 mM) was added to each tube, in order to stop DNAse reaction, and left for 5 minutes in Thermocycler (Applied Biosystems), at 65°C. Treated RNA was reverse transcribed using 1 µL OligoDT and 200 U M-MLV per sample, returning to the Thermocycler for 50 minutes at 37°C and 15 minutes at 70°C. The tubes were then stored at 4ºC. (de Lima et al., 2016a).

For RT-qPCR, all 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 for reference genes. The reaction was performed in a total volume of approximately 20 µL, containing: 10 µL 25-fold diluted cDNA template, 4.25 µL sterile water, 2 µL 10× PCR Buffer (Invitrogen), 1.2 µL of 25 mM $MgCl_2$ (Invitrogen), 0.1 µL of 10 mM dNTP (Invitrogen), 2 µL SYBR Green (1:10,000, Molecular Probes, Applied Biosystems), 0.2 µL of each of the 10 µM forward and reverse gene-specific primers and 0.05 µL Platinum® Taq DNA polymerase (5 U/µL, Invitrogen). Reactions were performed in a StepOne™ Real-Time PCR System (Applied Biosystems). After a phase of denaturation of 5 min at 95°C, the amplifications were carried out with 40 cycles of 15 s at 95°C, 10 s at 60°C, and 15 s at 72°C. Samples were held for 2 min at 40°C for annealing of the strands, and then heated from 55°C to 95°C with a ramp of $0.5°C.s^{-1}$ to generate the melting curves. 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 (Rujiter et al., 2009). PCR average efficiency was determined for each amplicon.

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

	Gene	Function	Primer sequence (5'-3') (forward/reverse)	Amplicon length (bp)
Pc2TPS	**(−)-β-pinene synthase**	(-) β-pinene synthesis	GAGCTTCTCAAACCCGACAG/ GGAGGGTTCTCATCACCAAA	148
Pt2TPS	**(+)-α-pinene synthase**	(+)α-pinene synthesis	CGACAACATCAACGAAATGG/ TCGAACGTGGGAAGATAACC	147
Pc1TPS	**(+)-α-pinene synthase**	(+)α-pinene synthesis	AGGTTGCCTACGGATGTCAG/ TGGTATCTTCTATGCTCCGAATC	101
Pt3b	**α-farnesene synthase**	α-farnesene synthesis	TGGGAAGCTTTAATCGATGC/ GGAGAGTGGCTGCTCGATAC	124
Pc3a	**abietadiene synthase**	abietadiene synthesis	GAATGCTCTGGAGGATACGG/ TCCAGCCTTGGCATACTTCT	114
ERF112	ethylene-responsive transcription factor ERF112	Transcription factor that active gene expression in response to ethylene	TACAGAGGCGTAAGGCAGAG/ CGACTTCCCCTGAATCTCAA	152
GGDS	geranylgeranyl diphosphate synthase	Geranylgeranyl diphosphate synthesis	CTGGCAGTGAATGAGGCTCT/ ACAAGAGGACGAATCCGTTT	113
CYP736B	cytochrome P450 monooxygenase	May be involved in fungal resistance	CCGTTGTGGTTTCTTCACCT / GATTCTCCTGGCAGTCAACAG	197
HISTO3	histone 3	Cell proliferation, DNA Binding, RNA methylation	GCTGAGGCTTACCTTGTG/ CCAGTTGTATATCCTT AGGCATAA	94
UBI	ubiquitin	Signaling complexes for protein degradation, translation control, DNA repair, endocytosis regulation, protein traffic	GATTTATTTCATTGGCAGGC/ AGGATCATCAGGATTTGGGT	149

2.4 Brachyblast resin gravimetry

Resin gravimetry was performed to verify whether there was correlation between different phenotypes and resin extracted from individual brachyblasts. Brachyblasts from different portions (top, middle and bottom) of each individual tree were collected, weighed and left for 24 h in - 4°C. Thereafter, the plant parts were cut in approximately 0.5 cm pieces, to which 5 ml of hexane were added for every 3 g of plant biomass. The samples were then sonicated for 20 min to promote cell-wall disruption and dissolution of the resin in the solvent. Samples were maintained for 24h at room temperature and the resulting material was placed in syringes and pressurized by a plunger, in order to retrieve as much resin as possible. Samples were left in a fume hood for 24 hours, and resin was quantified by its weight.

2.5 Statistical analyses

After normal distribution evaluation, results were statistically compared by unpaired t-test or Mann-Whitney test, using the software GraphPad Prism 7 (reference). Standard *P* was 0.05.

3 – Results

3.1 – Microtapping

The microtapping method to evaluate resin production in young plants showed significant differences between the two phenotypes, confirming the potential of this procedure to correctly identify specimens with distinct resin yield (Fig. 1).

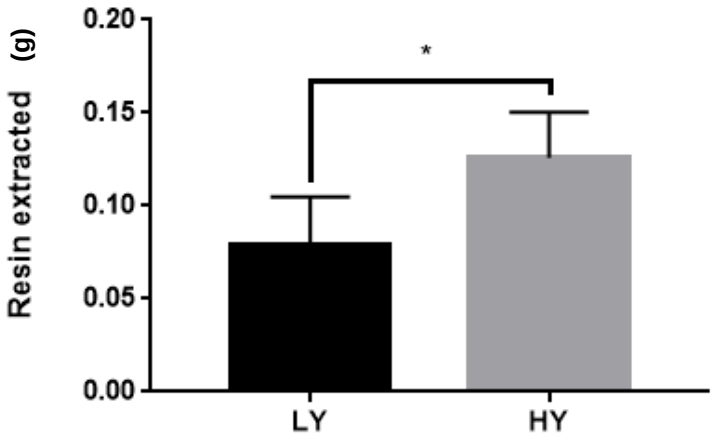

Figure 1. Resin extracted by the microtapping method. Bars represent mean of the 4 individuals of each phenotype, with standard deviation denoted by the lines above the bars. The asterisk indicates that the results were significantly different (P < 0.05). Data were compared by unpaired t-test (LY – low-yield individuals, HY – high-yield individuals).

3.1 – Gene expression

Pt2TPS and *Pc1TPS* genes (data not shown and Fig. 2a, respectively), which code for α-pinene synthase, showed a similar pattern of higher

expression in low-yield resin phenotype individuals. *Pc2TPS*, a gene coding for β-pinene synthase, had the opposite profile, with higher expression in the high-yield phenotype individuals (Fig. 2b). The transcript of *Pt3b* gene (coding for farnesene synthase) was not amplified at all in high-yield individuals, but showed consistent amplification in the low-yield ones, in spite of the elevated variability (Fig. 2c). On the other hand, *Pc3a*, which codes for the enzyme abietadiene synthase, was not amplified in the low-yield individuals and had extremely low levels of expression in high-yield ones (Fig. 2d).

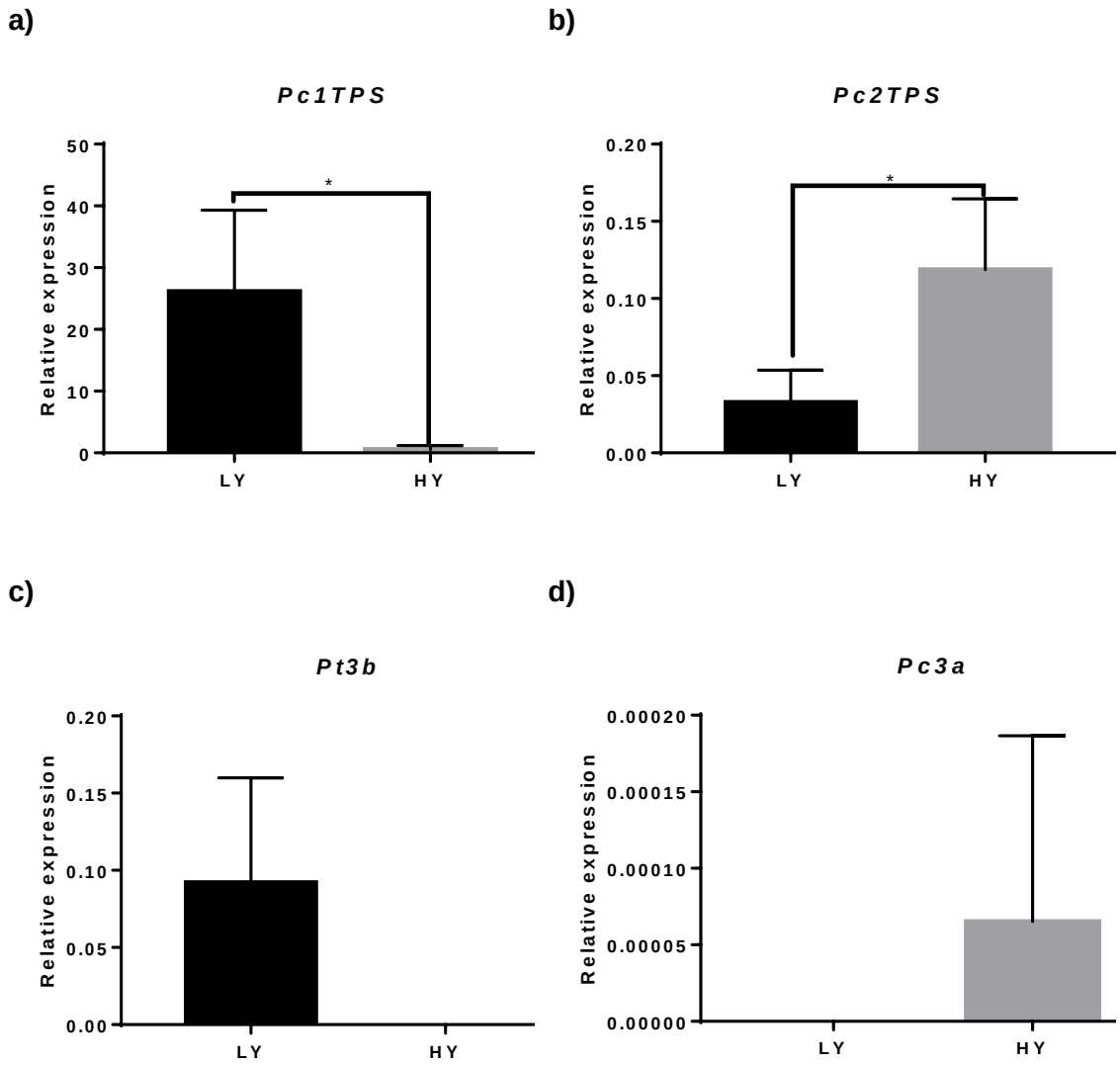

*Figure 2. Relative expression of Pc1TPS (**a**), Pc2TPS (**b**), Pt3b (**c**) and Pc3a (**d**). Bars represent mean of the 4 individuals of each phenotype, with standard*

deviation denoted by the lines above the bars. The asterisk indicates that the results were significantly different (P < 0.05). Relative expression was calculated relative to the reference genes HISTO3 and UBI. Data were compared by unpaired t-test (LY – low-yield individuals, HY – high-yield individuals).

GGDS gene codes for geranylgeranyl diphosphate synthase, which catalyzes the synthesis of GGPP in the early stages of diterpene biosynthesis. There was no significant difference in expression of *GGDS* between low and high-yield individuals (Fig. 3).

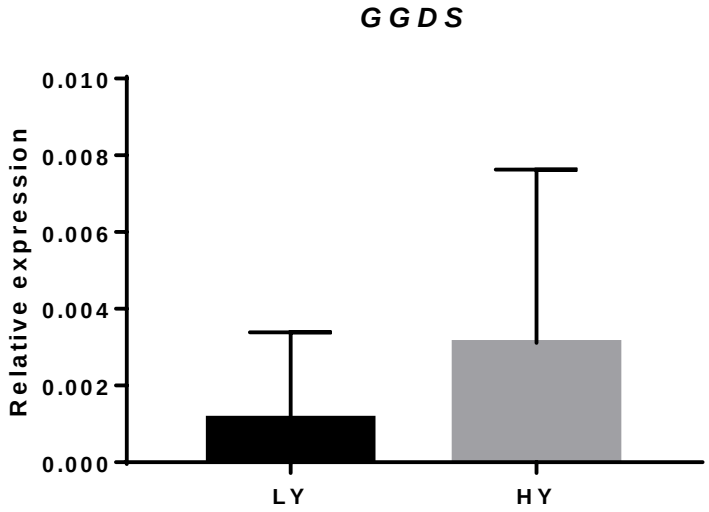

Figure 3. Relative expression of the GGDS gene, involved in the biosynthesis of geranylgeranyl diphosphate. Bars represent mean of the 4 individuals of each phenotype, with standard deviation denoted by the lines above the bars. There was no significant difference (P < 0.05). Relative expression was calculated relative to the reference genes HISTO3 and UBI. Data were compared by Mann-Whitney test (LY – low-yield individuals, HY – high-yield individuals).

The *ERF112* gene encodes a transcription factor that activates ethylene mediated gene expression. The expression of this gene was significantly higher in the high-yield phenotype individuals (Fig. 4).

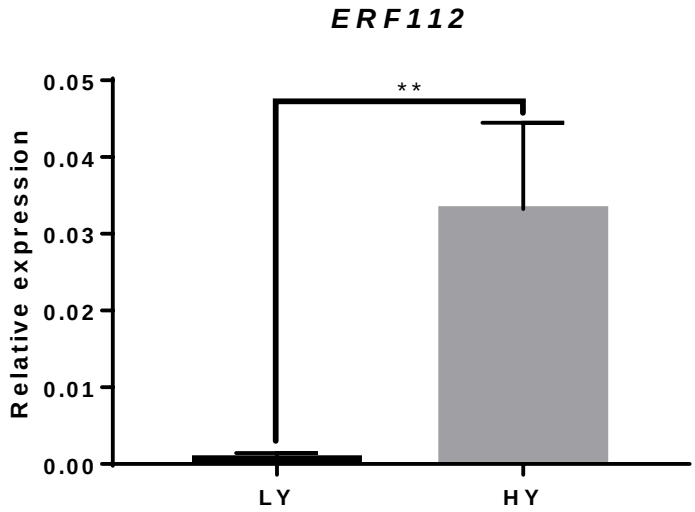

Figure 4. Relative expression of the ERF112 gene (transcription factor that activates gene expression in response to ethylene). Bars represent mean of the 4 individuals of each phenotype, with standard deviation denoted by the lines above the bars. The asterisk indicates that the results were significantly different (P < 0.05). Relative expression was calculated relative to the reference genes HISTO3 and UBI. Data were compared by unpaired t-test (LY – low-yield individuals, HY – high-yield individuals).

Additionally, *CYP736B*, a gene that codes for a cytochrome P450 monooxigenase, was evaluated. However, both phenotypes had undetectable expression in two or more individuals, and therefore statistical comparison could not be carried out.

3.2 – Resin gravimetry

Although a relatively higher amount of resin was extracted from the high-yield phenotype individuals, no significant difference was found. Again, the results showed high variability among replicates (Fig. 5).

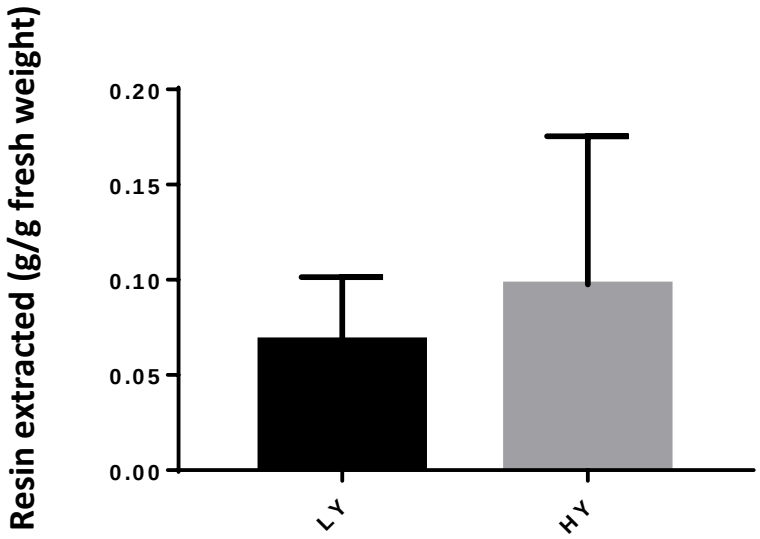

Figure 5. Quantity of resin extracted per gram of fresh weight determined by the gravimetric methodology. Bars represent mean of the 4 individuals of each phenotype, with standard deviation denoted by the lines above the bars. There was no significant difference between treatments (P < 0.05). Data were compared by unpaired t-test (LY – low-yield individuals, HY – high-yield individuals).

4 – Discussion

Taken together, data indicated a good correlation between the resin collected by microtapping of the trees and gene expression profiles. Gene expression data also shed some light on resinosis mechanisms.

Resin extracted from the individuals by microtapping at 3 years of age confirmed the previously determined yield phenotype obtained with 1 year-old saplings. Ferreira et al. (2011) demonstrated that one-year seedlings of *P. elliottii* can modulate resin production when challenged by environmental stimuli, such as flooding. In the present study, methyl jasmonate, which is an important signaling molecule for plant defense (Lombardero et al., 2013; Moreira et al., 2009), was used to induce resin exudation in the wound. The methodology of microtapping was further validated as a tool to precociously identify the seedlings worth planting for resin purposes, potentially improving the overall yield of forests derived from them.

It is not only the genetic difference between species and individuals that alters the yield and composition of terpenes in pine resin (Lundborg et al., 2016). As a number of studies asserted, besides genotype, the type and intensity of environmental stresses, can modulate terpene composition (Niinemets et al., 2013).

Pinenes (α and β pinene) are the main monoterpenes in slash pine turpentine. These metabolites are highy versatile, being used in the chemistry industry to produce oil, food, flavors and fragrances (Rodrigues-Corrêa et al., 2011; Sukarno et al., 2015). In our findings, the α-pinene synthase genes had significantly higher expression in low-yield individuals, whereas the gene coding for β-pinene synthase had greater expression in the high-yield individuals. These findings were unexpected, since pines tend to have a higher α-pinene/β pinene ratio, and the differences in this proportion is mostly noticed when comparing different parts of the plant (Rodrigues et al., 2011). However, auxin (naphthalene acetic acid) stimulation of resin tapped slash pine adult trees caused the ratio to favor beta pinene versus alfa (Neis et al., 2018). The expression of α-pinene synthase in the low-yield individuals was much higher than in the high-yield ones, suggesting different capacities to synthesize resin monoterpenes in the two phenotypes. It is noteworthy that a greater β-pinene/α-pinene ratio has a higher market value. Overall, pinene content composition and related gene expression may be indicatives of resinosis capacity in different individuals.

The *Pt3TPS* gene, coding for a farnesene synthase, was expressed in the low-yield individuals, but had no amplification in the high-yield ones. This suggests that high resin yielding individuals invest more carbon in mono and diterpenes compared to sesquiterpenes. Karanikas et al. (2010), studying resin composition of *Pinus halepensis* Mill. in two different localities, with a control group and a high resin yield, found significant increase in the percentage of the majority of sesquiterpenes in the individuals which produced more resin, in both localities. The same findings were reported for *Pinus massoniana* Lamb (Liu et al., 2015). These contrasting findings between gene expression and metabolite accumulation may reflect the role of other levels of regulation, such as protein synthesis, post translational changes, and availability of enzyme cofactors and

activators (de Lima et al., 2016a) or age and/or species differences in biosynthetic profiles.

Diterpenoids are synthesized from GGDP, formed by sequential condensation reactions of IPP and DMAPP (Zulak and Bohlmann, 2010). They are also the main components of the non-volatile fraction of resin. It was tested if *GGDS* gene, coding for the enzyme responsible for GGDP production, and the gene *Pc3aTPS*, involved in coding for abietadiene synthase, had different expression profiles in the two tree phenotypes. Expression of *GGDS* was did not differ between resin yield phenotypes. *PC3aTPS* was not amplified in the low-yield individuals, and had very low and variable levels of expression in high-resin yield ones. Karanikas et al. (2010) found an overall reduction in diterpenes proportion in highly productive *P. halepensis* individuals. Also, abietadiene showed only a slight increment in composition for these individuals in one location, although it mantained the same concentration in the other study area. However, the study of Liu et al. (2015) observed an opposite trend for *P. massoniana*. High-yield individuals had reduced concentration of diterpenes in resin, although the expression of the *GGDS* gene was much higher in these individuals. Again, these results indicate that there may be species-specific profiles or they could reflect differences in age and site of evaluation, as well as the participation of post-transcriptional regulation, as mentioned above.

Expression of the gene *ERF112* coding for an ethylene-responsive transcription factor was also evaluated. Ethylene plays a major role in the response of plants to the most diverse arrays of biotic and abiotic stresses (Khan et al., 2017). With regards to resin production in conifers, the gaseous phytohormone enhanced 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). In our study, samples of plants from the high-yield phenotypes displayed much higher expression compared to the low-yielding individuals. This result is in good agreement with the findings of Liu et al. (2015).

We also tested the expression of *CYP746B*, a gene coding for a cytochrome P450 monooxygenase. This class of enzymes is fundamental for the oxidation of products of diterpene synthases to yield resin acids (Ro et al., 2005 apud Chen et al., 2011). However, we could not detect expression in a sufficient number of individuals to be able to compare them.

Brachyblast resin gravimetry was evaluated because of its potential as an inexpensive and simple procedure for detecting an individual's promptness to produce higher quantities of resin relative to other trees (Moreira et al., 2009). Nonetheless, gravimetry analysis showed no significant difference between the tree groups. This may be due to the young age of the plants used in this study. Further investigations are required to verify if there could be different quantities of extracted resin from a larger number of young individuals, and also from adult pine trees, using this methodology.

5 – Conclusion

It was possible to distinguish the phenotypes of plants through two methodologies used in this work. Notably, even at young life-stages, plants already expressed their different resin synthetic potential. Differential amplification of some genes showed that high-yield and low-yield phenotypes can be distinguished by the expression of terpene synthases genes and the tested ethylene-responsive transcription factor gene. These sequences may be used to assist breeding and selection of slash pine trees aiming at the establishment of elite resin tapping plantations. The gravimetric analysis showed no dissimilarities among phenotypes, but a refined methodology can be promising to further attest resin production potential in young plants.

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