

Seasonality and chemical elicitation of defense oleoresin production in field-grown slash pine under subtropical climate

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ABSTRACT: Oleoresin is a key defense strategy of advanced gymnosperms, based on the combination of a complex anatomical structure of resin ducts and elaborate terpene biochemistry. Given the vast array of oleoresin economic applications in the chemical, pharmaceutical, agrochemical, and biofuel industries, translating factors that regulate terpene biosynthesis into higher oleoresin yield is a challenge for the forestry industry. Field tests with approximately 3,500 28-year-old slash pine (*Pinus elliottii* Engelm. var. *elliottii*) trees were carried out from 2005 to 2008, under the subtropical climate of Southern Brazil, in order to examine the seasonal profile of oleoresin production stimulation in response to different chemical adjuvants, after mechanical injury. Yields of trees treated with oleoresin-inducing pastes containing alternative adjuvants were compared to the standard commercial one used on an industrial scale (based on the ethylene-releasing compound – 2-chloroethylphosphonic acid - CEPA). Significant increases in pine oleoresin yield were observed by modulating its biosynthesis and using chemical stimulants affecting defense responses (benzoic acid, used in addition to CEPA) and biosynthetic enzymes (metal cofactors of terpene synthases, iron or potassium, used as replacements for CEPA). Oleoresin stimulation was consistent over at least four consecutive years. Overall effectiveness of oleoresin yield adjuvant stimulation was higher in the faster growth seasons, although potassium was effective in all of them. Combining metal cofactors did not show synergistic or additive interactions. The results suggest that higher oleoresin yields can be obtained by using individual adjuvants of the same signaling pathway in a season-specific fashion.

KEYWORDS: mechanical wounding, benzoic acid, metal cofactors, potassium, iron, terpene synthases, resin tapping.

INTRODUCTION

Slash pine (*Pinus elliottii* Engelm.) is characterized by the production of substantial amounts of oleoresin, which is a renewable source of terpenes, the largest class of plant secondary metabolites biosynthetically derived from isopentenyl diphosphate – IPP (Rodrigues-Corrêa et al. 2012). Briefly, terpenoid biosynthesis initiates with the formation of IPP (or its isomer DMPP – dimethylallyl diphosphate) from mevalonate (in the cytosolic pathway) or from MEP (2-C-methyl-D-erythritol 4-phosphate), in the plastidic pathway.

Following the formation of the basic hemiterpenoid building blocks, other IPP units are added by prenyltransferases (or IDS – short-chain isoprenyl diphosphate synthases) (Schmidt et al. 2011). This is done to synthesize geranyl diphosphate – GPP (C_{10}) and geranyl GPP (C_{20}) (mostly in plastids) and farnesyl diphosphate – FPP (C_{15}) (mostly in cytosol), acyclic precursors of mono, di, and sesquiterpenes, respectively (Huber et al. 2004, Schmidt et al. 2011). The final steps of terpenoid biosynthesis (cyclization of the acyclic precursors) are catalyzed by the terpene synthases (TPS) or cyclases, which are enzymes capable of generating

unique or multiple terpenoid products (Hall et al. 2013). TPS require divalent ions for catalysis (Huber et al. 2004, Keeling and Bohlmann 2006), and can have their activity improved by some monovalent cations in gymnosperms (Savage et al. 1994).

Besides the product versatility displayed by the TPS, the wide diversity and chemical complexity of oleoresin terpenoids may be also attributed to alternative modifying reactions including methylations, oxidations, and additional prenylation events. Studies carried out with different conifer species have shown that TPS are able to respond to different stimuli by modulating both quality (mono, sesqui, and diterpenes composition profiles) and quantity of the oleoresin produced, according to the type of stimulus perceived (McKay et al. 2003). Furthermore, members of the multi-gene TPS family have shown to be wound-induced (McKay et al. 2006), and upregulated by insect attack (McKay et al. 2003) and methyl jasmonate (MeJA) exogenous application (Fäldt et al. 2003). The exogenous application effects of defense-related chemical elicitors, such as salicylic acid (SA) and MeJA, on conifer defense metabolism, have been investigated in *Pseudotsuga menziesii*, *Sequoiadendron giganteum* (Hudgins and Franceschi 2004), *Pinus elliottii* (Rodrigues and Fett-Neto 2009), and *Picea abies* (Krajnc et al. 2011), showing variable responses depending on the species examined.

Pine oleoresin represents the major defense mechanism of conifers against their predators, and it may be either constitutively biosynthesized and stored in specialized structures, the resin ducts (RD) (preformed oleoresin) or its biosynthesis can be induced by external challenges as a characteristic defense response (inducible oleoresin) that takes place, for example, after traumatic resin duct formation (McKay et al. 2003, Phillips 2012). Commercially, both oleoresin main fractions (turpentine and rosin) exhibit several applications in chemical industries, including the synthesis of solvents, perfumes, waterproof materials, paper sizing, printing inks, food additives, among others (Rodrigues-Corrêa et al. 2012), being of special interest the oleoresin produced by slash pine due to its physicochemical properties (Rodrigues-Corrêa and Fett-Neto, 2012).

Biotic and abiotic stresses, including mechanical injury, pathogen attack, water availability, seasonality, and chemical stimulating applications, for instance, have been reported as key factors affecting pine oleoresin biosynthesis (Lombardero et al. 2006, Rodrigues and Fett-Neto 2009, Ferreira et al. 2011). Most resin-stimulating pastes are based on a mixture of pyrogenic silica, emulsifier, and active ingredients such as sulphuric acid and 2-chloroethylphosphonic acid (CEPA), an ethylene-releasing compound, at 20 and 4.5% w/v, respectively (Rodrigues et al. 2011). In order to further increase oleoresin yield in slash pine forests, the current

commercial production of oleoresin biomass in Southern Brazil has been based on physiological responses previously observed by modulation of some factors, and by testing the application effect of resin stimulating pastes with different chemical formulations (Rodrigues et al. 2008, Rodrigues and Fett-Neto 2009, Rodrigues et al. 2011). Furthermore, alternative chemical elicitors capable of replacing CEPA, which represents the highest cost for oleoresin producers in resin tapping operations (Rodrigues et al. 2011), have also been actively sought. However, these studies have been done essentially on a whole-year oleoresin harvest basis. The present study was focused on the effects of alternative chemical elicitor exogenous applications on seasonal oleoresin production in slash pine, compared to trees treated with commercial paste containing CEPA, in consecutive years.

MATERIAL AND METHODS

All trials were carried out in field-grown 28-year-old slash pine forests cultivated in Southern Brazil, which were not previously tapped (Rio Grande do Sul state, city of São José do Norte, approximately 32° of south latitude and 52° of west longitude). Trees were tapped biweekly (except in “frequency test”), according to the bark streak (Pio and Valente 1998, Rodrigues et al. 2008) or the American method (Cunningham 2012) that removes the bark, phloem, and vascular cambium tissues to expose the sapwood, surface where the chemical stimuli (stimulating pastes) are applied.

The seasonal oleoresin production was evaluated from 2005 to 2008 in metal cofactors tests. At the end of each season, the plastic bags attached to the trees were collected and weighed on a digital balance, after disposing the top water carefully. Seasons for these assays were limited to the following monthly distribution: Winter (June – August), Spring (September – November), Summer (December – February), and Fall (March-May). The performance of the commercial paste (20% H₂SO₄ and 4.5% CEPA), used in large scale in Southern Brazilian resin tapping operations (Rodrigues and Fett-Neto 2009), was compared with that of pastes containing TPS metal cofactors (K, Fe, Mn) or ethylene receptor (Cu) ones (Savage et al. 1994, Rodrigues et al. 2011), either isolated or combined in the absence of CEPA, and pastes with benzoic acid (BA), which is a SA precursor (Shah 2003), in the presence of CEPA.

The frequency effect of the commercial paste application on oleoresin yield was also evaluated.

Potassium: In order to assess the effect of potassium on CEPA replacement, 550 individuals per treatment (paste containing 500 mol m⁻³ of K₂SO₄ [potassium sulfate] without CEPA versus commercial paste – Control) were tested in two different sites, totaling 2,200 trees tested.

Iron : In the same way, iron effect was evaluated by testing 550 trees per treatment (paste containing 10 mol m^{-3} of $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ [ferrous sulfate] without CEPA *versus* commercial paste – Control).

Combined metal cofactors: The TPS metal cofactors K^+ (500 mol m^{-3}), Fe^{2+} (10 mol m^{-3}), Mn^+ (1 mol m^{-3}) (provided as MnSO_4), along with the ethylene receptor component Cu^{2+} (1 mol m^{-3}) (provided as CuSO_4), were combined in two different ways for the purpose of observing a possible synergistic or additive effect in the oleoresin production.

Benzoic acid: Two hundred trees were evaluated per treatment (commercial paste containing 10 mol m^{-3} of BA *versus* commercial paste – Control).

Frequency of wounding and commercial paste application: The influence of the chemical stimulus frequency applied on the bark streak was measured in three treatments: mechanical wound only (bark streak); application of the commercial paste every 28 days; application of the commercial paste every 14 days. One hundred individuals were tested per site. All trials were performed in two places and the results are presented as the mean values of both locations. In metal cofactors and BA tests, the data were analyzed by using the Student's *t*-test comparison of treatments *versus* control samples, with $p \leq 0.05$. Analyses of variance (ANOVA) followed by Dunnett's C were used for data evaluation in the wound frequency test.

RESULTS AND DISCUSSION

Significant differences were observed in the frequency test of commercial paste application. As expected, considering the stimulant effects of the SA and CEPA on oleoresin production (Rodrigues and Fett-Neto, 2012), slash pine trees showed higher annual oleoresin yields when commercial paste was applied on the bark streak biweekly ($3.666 \pm 0.683 \text{ kg}$), compared to monthly application ($2.749 \pm 0.395 \text{ kg}$) and no chemical elicitor application ($1.632 \pm 0.267 \text{ kg}$). Increases between 1.5 to 2.5 fold in oleoresin yield by chemical stimulation compared to wounding alone were often observed in various field experiments (Rodrigues et al. 2011). Several reports have shown the effect of abiotic factors on oleoresin biosynthesis and monoterpene emissions. In slash pine (Tingey et al. 1980) and *P. halepensis* (Peñuelas and Llusà 1999), the rates of monoterpene emissions were enhanced with temperature increase. Temperature oscillations were also shown to influence oleoresin production of four pine species tested in Brazilian stands. *Pinus elliottii* var. *elliottii*, *P. oocarpa*, *P. caribaea* var. *bahamensis*, and *P. kessya* trees, tapped during Spring and Summer, had inducible oleoresin yields reduced during the lowest temperatures registered (Brito et al. 1978).

Monoterpene emissions have exhibited a seasonal pattern. In their seasonal evaluation from three coniferous spp. (*Cryptomeria japonica*, *Chamaecyparis obtuse*, and *P. koraiensis*), the highest ones were observed in Spring and Summer (Kim et al. 2005). Comparable results were found for *P. densiflora* growing in East Asia (Lim et al. 2008) and *P. sylvestris* growing in Southern Germany (Holzke et al. 2006). In different regions; however, it has exhibited a variable profile of monoterpene emissions, displaying higher concentrations in early Summer (pure Scots pine stand) (Räisänen et al. 2009) and in Summer and Fall (increasing in association with some spruces, aspen, and birch) (Aaltonen et al. 2011) in Eastern and Southern Finland, respectively.

In our tests, a seasonal response was also seen for paste supplemented with Fe^{2+} . Total replacement of CEPA by this metal was shown to be possible only in Spring and Summer (Figure 1). In *P. taeda* treated with fungal inoculation, the induced oleoresin average flow was higher in Summer when compared to Spring (Tisdale et al. 2003). The authors suggest that this pattern may be correlated with the early wood and latewood formations, which occur in Spring and Summer, respectively, and they would modulate the photosynthate availability for oleoresin formation. However, previous experiments carried out in subtropical climate have shown to be Spring the most productive season in terms of constitutive and inducible oleoresin productions (Rodrigues and Fett-Neto 2009), except in extreme conditions. For example, oleoresin yields in slash pine treated with different chemical pastes were higher in Winter than in Summer in the year 2005 (Rodrigues and Fett-Neto, unpublished results). This abnormal production was more severe in extremely dry sites and years. Hence, water availability may also be a decisive factor affecting oleoresin biosynthesis.

The activity of constitutive monoterpene cyclases was reduced by water (and light) stress in *Abies grandis* (Grand Fir) saplings and mature trees. Furthermore, there was an interruption of wound-induced response (oleoresinosis) caused by the dry condition (Lewinsohn et al. 1993). Transient flooding treatment enhanced the oleoresin yield of one-year-old slash pine seedlings in greenhouse conditions (Ferreira et al. 2011). Irrigation tests performed on *P. sylvestris* L. dry sites showed a positive correlation between radial growth and moisture in Spring, albeit resin duct formation had been stimulated mainly by high Summer temperatures (Rigling et al. 2003).

In annual terms, potassium treatment presented superior yields of oleoresin along the four-year evaluation compared to commercial paste, being the last one the most productive year. Interestingly, the positive effect of potassium was observed throughout the seasons of the years examined (Figure 2). This stimulation was especially conspicuous in the transiently

waterlogged site. Highest oleoresin production was also observed in the fourth year of resin tapping in slash pine for both faces of the trunk (Figueiredo-Filho et al. 1992). In the present tests, the increased oleoresin flow seen in the last year evaluated does not seem to be related to the differential density or size of constitutive RDs, since both parameters decrease with age (in slash pine the number of RDs reaches a stable level at approximately 20 years of age) (Hodges et al. 1981). Also, all trees tested were almost three decades old. These results confirm previous observations concerning the role of potassium as a suitable substitute of CEPA (Rodrigues et al. 2011) in stimulating paste formulations. Herein, we showed that, at least for the sites and conditions tested, potassium stimulatory effect on oleoresin yield as a replacement for CEPA in resin inducing paste was not strongly affected by season.

Previously, annual oleoresin production of 28-year-old slash pine trees was assessed by using isolated metal cofactors of TPS and ethylene receptors at two different sites (Rodrigues et al. 2011). Higher yields were obtained using pastes supplemented with low concentrations of copper (1 mol m^{-3}) (a component of ethylene receptors), moderate of iron ($10 \text{ or } 100 \text{ mol m}^{-3}$), and high of potassium (500 mol m^{-3}). In the present study, no synergistic effect on oleoresin biosynthesis was observed in pastes combining TPS metal cofactors. The absence of CEPA in pastes caused significantly lower oleoresin yields in the colder seasons, this difference becoming less pronounced in Spring (Figure 3). Moreover, efficient replacement of CEPA was observed only in the Summer regardless the presence of copper. These results suggest that there was no additive effect by combining metal cofactors involved in similar or correlated biochemical functions.

Similarly to what was observed in tests with iron, commercial paste containing BA in its formulation presented better performance as oleoresin inducer in Spring and Summer

than in Fall and Winter (Figure 4). Little is known about conifer physiological response to BA treatment. However, there are few works concerning the effects of SA exogenous application. Methyl salicylate ($10, 25, 50 \text{ and } 100 \text{ mM}$) treatment in *Pseudotsuga menziesii* and *Sequoiadendron giganteum* did not induce either oleoresin or phenolic biosynthesis, typical defense-related responses displayed by conifers (Hudgins and Franceschi 2004). Following SA treatment (10 mol m^{-3}), slash pine showed superior oleoresin yields when compared to controls (pastes with CEPA), and equivalent yields in pastes prepared without CEPA in all seasons tested (Rodrigues and Fett-Neto 2009). SA exogenous application (100 mM) in *Picea abies* inhibited the colonization by *Ips typographus*, possibly as a response mediated by the induction of antioxidant defense mechanisms (Krajnc, Kristl and Ivancic, 2011). Methyl salicylate, SA, and its precursor BA are involved in pathogen-defense mechanism signaling (Shah 2003) and possibly mimic the pathogen attack signals, by inducing oxidative stress and promoting the transcription of defense-related genes (Krajnc et al. 2011).

Albeit the effects of CEPA have not been entirely replaced by pastes supplemented with iron in all seasons tested, its application remains viable since most of it exhibited equivalent or superior yields compared to the commercial paste in Spring and/or Summer. Taking into account that they represent the main period of the year in which commercial resin tapping operations are carried out, and the significantly higher market price of CEPA, its replacement by means of the development of seasonal (metal cofactors-based) pastes would provide a significant gain for resin tapping activity. The use of BA as an additional adjuvant with CEPA is also viable in the faster growth seasons, improving yields.

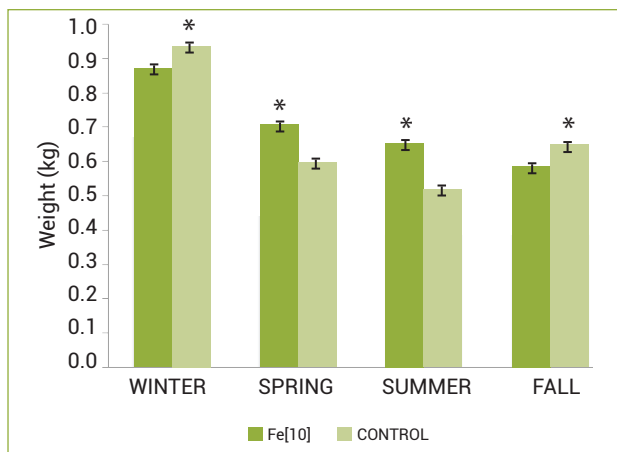


Figure 1. Seasonal variation of slash pine oleoresin production: iron (10 mol m^{-3}) effect as a full replacement of CEPA. Control paste is based on CEPA. Asterisks indicate the significant difference between treatments at a particular season.

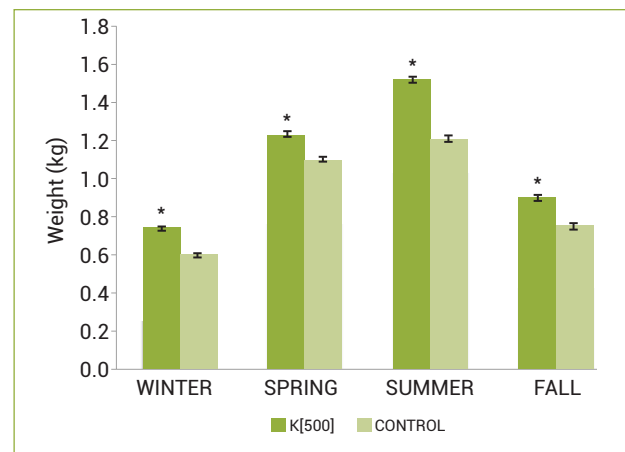


Figure 2. Seasonal variation of slash pine oleoresin production: potassium (500 mol m^{-3}) effect as a full replacement of CEPA. Control paste is based on CEPA. Asterisks indicate a significant difference between treatments at a particular season.

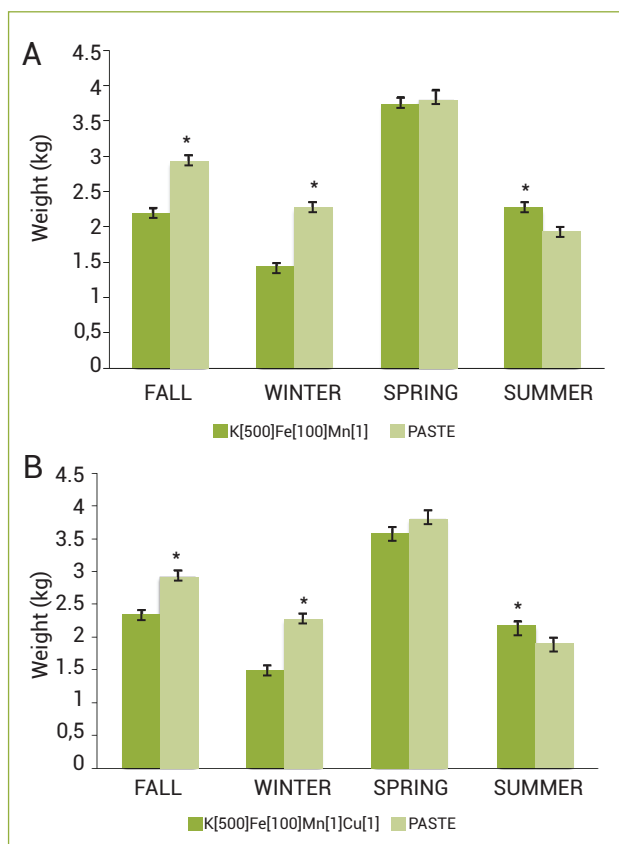


Figure 3. Seasonal production of slash pine oleoresin: combined metal adjuvant effects as a full replacement of CEPA. Control paste is based on CEPA. (A) Terpene synthases (TPS) metal cofactors combined (K 500 mol m⁻³ + Fe 100 mol m⁻³ + Mn 1 mol m⁻³). (B) TPS metal cofactors combined plus copper (K 500 mol m⁻³ + Fe 100 mol m⁻³ + Mn 1 mol m⁻³ + Cu 1 mol m⁻³). Asterisks indicate a significant difference between treatments at a particular season.

Therefore, data to date suggests that seasonal pastes could be designed to achieve the highest oleoresin yields with paste cost reduction, by replacing CEPA with metal adjuvants or combining it with BA, particularly in Summer and Spring.

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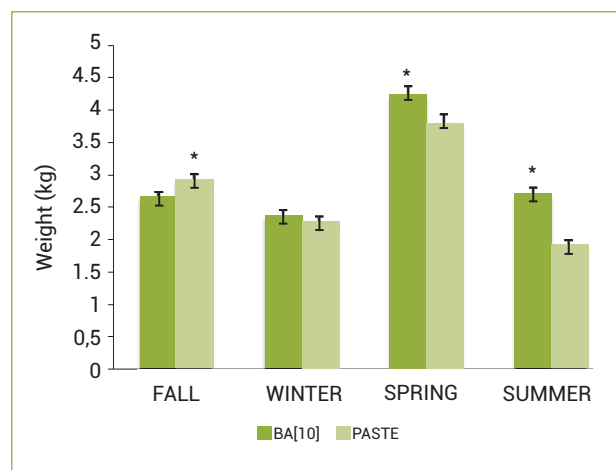


Figure 4. Seasonal variation of slash pine oleoresin production: benzoic acid effect as an adjuvant (10 mol m⁻³) added with CEPA. Asterisks indicate a significant difference between treatments at a particular season.

Further investigations are needed to verify if BA is capable of replacing CEPA, and to check for potential positive interactions between two different signaling pathways on oleoresin biosynthesis, such as BA (or SA) combined with one of the TPS metal cofactors.

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