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SHORT COMMUNICATION

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Salicylic and citric acid as promising new stimulants for resin tapping in maritime pine (*Pinus pinaster* Ait.)

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Abstract

Aim of study: We tested alternative active principles to the most widely used resin tapping stimulant which contains sulphuric acid. We also studied the effect of wounding in five-year-old *Pinus pinaster* seedlings with a microtapping method.

Area of study: The experiment was carried out at the Universidad Politécnica de Madrid in Spain.

Material and methods: The experiment consisted of six treatments: control (no stimulant no wounding), wound (no stimulant), and pines stimulated with sulphuric acid, ethrel, salicylic acid and citric acid. We evaluated the resin yield differentiating between released resin and internal resin (resin retained within the xylem), and the physiological status of the tree.

Main results: Wounded plants produced on average three times more resin than control plants. Plants stimulated with salicylic and citric acids showed the highest resin yield and produced on average 15% more resin than those stimulated with sulphuric acid, mainly because the released resin was higher. Tree diameter affected resin yield and thicker trees produced more resin. We did not observe any significant effect of the treatments on stomatal conductance and only a marginal significant effect ($p<0.10$) on water potential.

Research highlights: Salicylic acid and citric acid seem to be promising stimulants for the resin tapping activity to be further tested in field experiments with adult trees.

Keywords: sulphuric acid; ethrel; pine resin; microtapping; wounding; water potential; stomatal conductance.

Abbreviations used: TR: total resin content; RR: released resin; IR: internal resin; gs: stomatal conductance; Ψleaf: midday leaf water potential.

Authors' contributions: SM, ARG and RL conceived and design the experiment, SM, FR and RL performed the measurements, SM, ARG and RL analyzed the data, SM and RL drafted the manuscript, SM, ARG, LG and RL provided comments to the manuscript. ARG, LG and RL obtained funding and supervised the work.

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Introduction

Humans have been using resin since prehistoric times, but the system of resin extraction at an industrial level began at the beginning of 18th century. The first non-destructive method was developed in France and was known as the "Hugues method". It consisted on wounding the tree trunk deeply in the xylem every 5-6 days to release the resin contained in the resin canals. One of the main concerns of this technique from the beginning was that resin tend to crystallize quickly and thus the flow is considerably reduced and eventually stops. In cold regions this effect was more evident, thus in countries such as Germany or Russia, with extensive areas of pine forests, resin production was scarce. From the 1930s tapping related research in these countries focused on the development of chemical stimulants to increase resin flow and avoid crystallization. A few years later, new trials with stimulants also began in the USA (Nájera & Rifé, 1951). The results obtained were so promising that the Hugues method was progressively replaced by a less harmful method, "bark streak tapping", barely damaging the xylem and with the addition of the stimulant. This method increased profitability, reduced wood loss and required less effort for the resin-worker (Nájera, 1961).

In Spain, trials with stimulants began in the mid-20th century. After investigating different types of acids and bases the best results were obtained with sulphuric acid (Nájera & Rifé, 1953). During the first years, sulphuric acid was applied in a liquid state, but it entailed several problems for the worker's health and security. Moreover, the stimulant had to be reapplied every 6-7 days. To solve partly these issues, tests mixing the acid with clays and plasters were carried out to obtain a semi-solid paste (Zamorano & Solís, 1974). In Spain, the stimulant paste based in sulphuric acid, kaolinite ($Al_2Si_2O_5(OH)_4$) and calcium chloride was officially launched in 1969 and has remained practically the same until today. Its application is more easily controlled than in a liquid state and its reapplication period was extended to 14 days. Despite these advantages, the use of sulphuric acid is controversial due to three main reasons: safety for resin workers, very corrosive for pine trees and non-desired residue in the chemical industry. The effect of sulphuric acid in tree physiology is not known in detail. It seems to act prolonging the wounding effect and as a reactive oxygen species inducer, leading to a larger extension of the damage and consequent prolonging resin flow (McReynolds & Kossuth, 1982; Fett-Neto & Rodrigues-Corrêa, 2012).

Nowadays, commercial stimulant pastes in Spain are made of a mixture of sulphuric acid (60-20% v/v), and a carrier material, plaster, to provide adhesion on the wound. Newer formulas include Ethrel (also known as CEPA, 2-chloroethylphosphonic acid), an ethylene precursor that stimulates resin production and flow (McReynolds & Kossuth, 1982; Fett-Neto & Rodrigues-Corrêa, 2012).

In the last years, an extensive work is being devoted to the development of new formulas. New active principles include phytohormones such as auxins (Rodrigues *et al.*, 2008); salicylic acid (Rodrigues & Fett-neto, 2009) or its salt benzoic acid (da Silva & Fett-Neto, 2013) and jasmonates (Nogueira *et al.*, 2016). Adjuvants such as iron, potassium or copper have also been tested (da Silva & Fett-Neto, 2013).

The main objective in our study is testing alternative active principles to sulphuric acid in *Pinus pinaster* Ait., the main tapped pine species in the Mediterranean basin. We have used a standardized microtapping method to try to avoid the loss of volatiles, mainly monoterpenes (Trapp & Croteau, 2001). Besides the released resin, we have evaluated the internal resin, resin retained in the xylem of the piece of stem affected by microtapping.

Material and methods

Plant material and treatments

Five-year-old seedlings of *P. pinaster* growing in 3l pots with 70% peat and 30% sand (v:v) were used in the experiment. Plants have been fertilized with slow release fertilizer (Osmocote Plus Standard, 15:9:12 (N:P:K); 12–14 month release) once a year and watered at full capacity. At the beginning of the experiment, basal diameter of the trees ranged between 7.9 and 13.7 cm with an average diameter (±SD) of 10.7 ±1 cm. Trees were evenly distributed among treatments (Fig. S1 [suppl.]).

We used four resin stimulant pastes: i) traditional (74% sulphuric acid (50% v/v), 16% plaster), ii) ethephon (8% ethephon (60 % v/v), 14% sulphuric acid (50% v/v), 55% distilled water, 1.7% polysorbate, 1% cetyl alcohol, 4% vaseline, 5.5% silica, 10.8 sawdust), iii) salicylic (1% salicylic acid, 25% sulphuric acid (96% v/v), 5% propylene glycol, 19% wheat straw, 50% distilled water), and iv) citric (40% citric acid, 26% calcium bentonite, 0.5% propylene glycol, 500 g distilled water). Additionally, we included two other treatments, wounding without any kind of stimulant (wounded) and control plants without wounding or stimulant (control). For each treatment, 19 pine seedlings were used honing the diameter and height between each one. Thus the total number of pines used in the experiment was 114. The experimental design consisted in parallel rows north south direction. In each row we changed one position each treatment in order to homogenize light and watering.

The experiment lasted one month. In mid-August 2019, the bark and the cambium were removed at 10 cm-height

Figure 1. Detail of the wound filled with the stimulant (a) before sealing with a test tube and Parafilm for resin collection (b) (scale bars 5 cm).

with a punch of 1 cm of diameter, except in control plants. The hole was filled completely with the stimulant (Fig. 1a). To collect the resin, a previously weighed test tube was placed and sealed with the same portion of Parafilm tape (Fig. 1b). In mid-September, all the test tubes were collected.

Physiological measurements

In mid-September, needle stomatal conductance (gs) and water potential (Ψleaf) were measured at midday in 6 pines per treatment. Measurements were carried out in the upper part of the plant. Stomatal conductance was measured with a porometer (model SC-1, Decagon Devices Inc.) on three dwarf shoots per pine. Needles for water potential measurements were kept in plastic bags with a piece of wet paper at 4 ºC, until measurements were carried out in the laboratory with a pressure chamber (model 1000, PMS Instrument Company) within 2 h.

Resin yield

At the end of the experiment, the preweighed test tubes were collected and reweighed with resin and Parafilm. The net weigh of released resin was calculated subtracting the weight of the tube and the Parafilm (Released Resin, RR).

Resin retained inside the stem was extracted by slightly modifying the protocol described in Moreira *et al.* (2009). A 10-cm-long stem piece (5 cm above and 5 cm below the wound) was cut into small transversal discs (c. 5 mm width), transferred into preweighed test tubes and immediately frozen at -80 ºC until analyzed. Resin extraction was made covering the discs with hexane and inserting the tubes in an ultrasonic bath at 20 ºC for 15 min. Then the tubes were left for 24 h at room temperature under the fume hood. The extract was filtered through grade GF/F filters. The whole extraction step was repeated, and the solvent was evaporated to dryness under the fume hood at room temperature for approximately one week. The resinous residue was determined gravimetrically with a precision scale (0.0001 g) (Internal Resin, IR). The addition of RR and IR is defined as Total Resin content (TR).

Statistical analyses

The effect of wounding and the chemical stimulant on resin yield and physiological measurements was tested with a one-way ANOVA. The basal diameter of the plant was used as a covariate to include the effect of plant size in the model. Means were compared using Fisher's least significant difference tests (LSD, $\alpha = 0.05$). Pearson's correlation coefficients were obtained between internal, released, total resin and seedling diameter. Analyses were performed with R (version 3.4.2).

Results & Discussion

We did not observe any significant effect of the treatments on stomatal conductance (gs) and only a marginal significant effect (P<0.10) on water potential (Ψleaf). Stomatal conductance ranged between 234 mmol s^{-1} m^{-2} in the salicylic treatment and 287 mmol s^{-1} m^{-2} in the treatment with ethrel. Midday Ψleaf ranged between -0.8 MPa in the salicylic treatment and -1.4 MPa in the treatment with sulphuric acid. Thus, apparently wounding or wounding and stimulation did not affect much the water status of the tree (Table 1). Previous studies with *P. pinaster* showed that notching the stem decreased tree hydraulic conductance and reduce the availability of water for transpiration, affecting gs whereas Ψleaf remained almost unchanged (Ripullone *et al.*, 2007). In the same way, stem girdling resulted in a sharp decrease of gs even when the hydraulic conductance was increased (López *et al.*, 2015). In our experiment, however, neither wounding nor the application of stimulants resulted in lower gs, maybe due to the small damage in the stem or shorter duration of the experiment, and in the homeostasis of Ψleaf, commonly found in pines as typical isohydric species (Ripullone *et al.*, 2007).

Tree diameter affected resin yield and thicker trees produced more resin (Fig. S2 [suppl.]). This relationship was particularly evident for the internal resin (IR, r^2= 0.39, p<0.05; Fig. S3 [suppl.]). Our results are in accordance with previous studies with tapped adult trees in the field where vigorous trees with larger crown sizes or higher basal area increments showed higher resin yield (McDowell *et al.*, 2007; Rodríguez-García *et al.*, 2014). Bigger trees could easily mobilize the carbon stocks to defense systems for resin synthesis in good environmental conditions,

Table 1. Stomatal conductance (gs) and midday leaf water potential (Ψleaf) of tapped seedlings of *Pinus pinaster* one month after applying different chemical stimulants. Values are mean ± SE. There were not significant differences among treatments for stomatal conductance (gs) and only a marginal significant effect (P<0.10) on water potential (Ψleaf)

Treatment	gs (mmol s^{-1} m^{-2})	Ψleaf (MPa)
Control	259 ± 25	-1.0 ± 0.1
Wound	239 ± 30	-1.3 ± 0.1
Sulphuric	234 ± 27	-1.4 ± 0.1
Ethrel	303 ± 28	-1.1 ± 0.2
Salicylic	234 ± 26	-0.8 ± 0.1
Citric	236 ± 25	-1.1 ± 0.2

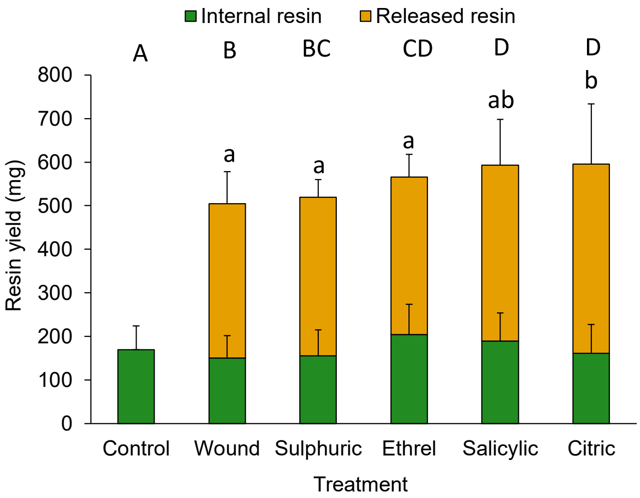

Figure 2. Internal (green) and released (yellow) resin yield in seedlings of *Pinus pinaster* tapped without treatment (Control) only wounded (Wound) with four stimulant pastes. Bars represent mean + SD. Identical capital letters correspond to homogeneous groups for total resin yield. Identical lower-case letters correspond to homogeneous groups for released resin. (LSD comparisons, $\alpha = 0.05$). Treatment was only marginally significant for internal resin (see the main text for more details).

where resin production is a function of the pool size of remaining carbohydrates (Ruel *et al.*, 1998; Lombardero *et al.*, 2000; Kane & Kolb, 2010).

We found significant differences between total resin content of control plants (no stimulant-no wounding) and wounded or stimulated plants (Fig. 2). Wounding seemed to play a key role in resin production regardless of the use of stimulants. Wounded plants produced on average three times more resin than control plants (556 mg on average of wounded plants compared with 170 mg of resin in control plants). These results could be the result of a systemic induced resistance which involve the formation of new resin canals (Moreira *et al.*, 2008) and the activation of constitutive axial resin canals (Krokene & Nagy, 2012; Rodríguez-García *et al.*, 2015, 2016). These systemic effects and terpenoid metabolism (Bonello & Blodgett, 2003) could result in increased resin flow (Lombardero *et al.*, 2000; Bonello *et al.*, 2006; Moreira *et al.*, 2009) and formation of new resin canals above the wound in the healing tissues (Chano *et al.*, 2015).

Total resin of plants stimulated with sulphuric acid was similar to TR in wounded plants and showed the lowest values. Whereas, plants stimulated with salicylic and citric acids showed the highest TR and produced on average 15% more resin than those stimulated with sulphuric acid. (Fig. 2). These results are in accordance with previous findings were higher resin productions were observed for salicylic acid-based stimulant treatments (Rodrigues & Fett-Neto, 2009; de Oliveira *et al.*, 2019). Salicylic acid is a signaling molecule of defense in plants, particularly against pathogens, and activate the defense system (Shah, 2003). However, its effect on resin yield seems to be species specific and de Oliveira *et al.* (2019) did not find any effect of salicylic acid in *Pinus elliottii* seedlings of similar age than the ones in our study.

Released resin (RR) was also higher in the treatments based on citric acid and salicylic acid (Fig. 2). Pines stimulated with citric acid released 22% more resin than only wounded pines and 19% more than pines stimulated with sulphuric acid (Fig. 2). From an economic point of view, RR is determinant. Even if TR is similar with certain treatments, the resin yield in the field (RR) can be lower, as happens in the treatment with ethrel in our experiment (Fig. 2). In this sense, the citric-based stimulant shows the highest TR and also the highest RR.

We found only a marginal significant difference ($p = 0.11$) among treatments for internal resin (IR). Ethrel was the treatment with higher resin accumulation inside the trunk. When we considered the ratio IR:TR, more than 35% of TR remained with ethrel *vs.* 27% with citric acid and 29% with sulphuric and wounded. Ethrel is known to release ethylene when it comes into contact with tissues and exceeds pH 3.5 (Fusatto, 2006). Ethylene triggers an increase in the synthesis of resin canals (Perotti *et al.*, 2015) that can increase resin synthesis (Fett-Neto & Rodrigues-Corrêa, 2012). Nevertheless, the resin produced could not be as easily released as with the citric or the sulphuric pastes. A significant increment of IR in the heartwood has also been reported in pines using methyl viologen, a powerful herbicide (Brown *et al.*, 1976).

The lack of differences between resin yield in the wounded-only and plants stimulated with sulphuric acid compared with actual values of resin yield obtained in the field with adult trees, suggests that microtapping may be used carefully for faster, less costly and precocious selection of adjuvants with potential high resin induction capacity in adult plants. It is important to highlight that plant age, timing and duration of the experiment influence the stimulant effects. In this sense, young conifer tests to evaluate resin production with different stimulants usually show less differences than treatments with older plants (de Oliveira *et al.*, 2019). This different response could be partly explained by the fact that young plants have more active basal metabolism and a faster response to wounding than adult plants, with a great investment in protein synthesis, growth and tissue formation (Gershenzon, 1994), and as a consequence, less investment in carbon allocation to resin. Furthermore, larger and more complex network of resin ducts in adult trees could justify higher sensitivity to chemical elicitors than the smaller one present in seedlings and samplings (de Oliveira *et al.*, 2019). However, the increments in total resin and released resin observed for salicylic and citric-based stimulants in our study may indicate that the effect of the most powerful adjuvants could be observed at early ages.

Although salicylic and citric acids look promising, further research is needed to: i) study the effect of repeated wounding on the trees, ii) assess the long-term effect of tapping on forest health and individual tree status iii) testing citric in bigger and older plants to see if they experience the same effects.

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