Chapter 2

Stimulant Paste Preparation and Bark Streak Tapping Technique for Pine Oleoresin Extraction

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Abstract

Tapping technique comprises the extraction of pine oleoresin, a non-wood forest product consisting of a complex mixture of mono, sesqui, and diterpenes biosynthesized and exuded as a defense response to wounding. Oleoresin is used to produce gum rosin, turpentine, and their multiple derivatives. Oleoresin yield and quality are objects of interest in pine tree biotechnology, both in terms of environmental and genetic control. Monitoring these parameters in individual trees grown in the field provides a means to examine the control of terpene production in resin canals, as well as the identification of genetic-based differences in resinosis. A typical method of tapping involves the removal of bark and application of a chemical stimulant on the wounded area. Here we describe the methods for preparing the resin-stimulant paste with different adjuvants, as well as the bark streaking process in adult pine trees.

Key words Oleoresin, Pine, Tapping, Chemical stimulant, Wounding

1 Introduction

Several conifer species produce oleoresin, a complex mixture of isoprenoid compounds relevant for defense against herbivores and pathogens. Two major fractions can be recognized in oleoresin: (a) turpentine, the volatile fraction containing mono- and sesquiterpenes, and (b) rosin, the nonvolatile diterpene fraction. Oleoresin is a forest commodity of global interest, finding applications in diverse industry sectors. Rosin is used in adhesives, printing ink manufacture, and paper sizing. Turpentine can be used either as a solvent for paints and varnishes, or as a raw material for fractionation of high-value chemicals used in the pharmaceutical, agrochemical, and food industry [1–3].

During the extraction activity, resin is obtained from the tree in a similar way as rubber tree tapping, which generally involves the

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preparation of the face of the tree, the installation of the resin collection system, tree wounding to induce resin flow, application of a chemical formulation to stimulate and maintain resin flow, collection of the resin, re-wounding of the tree, and application of the stimulant at suitable intervals [4].

Mechanical wound by itself induces defense responses in injured plants, but resin production can be chemically induced and modulated by applying stimulating pastes. Over the last 10 years, research has been conducted aiming at optimizing the production of induced oleoresin by the modification of the commercially used paste, which is basically composed of sulfuric acid or paraquat, *N*,*N*′-dimethyl-4,4′-bipyridinium dichloride (both involved in generating reactive oxygen species, resulting in increased and prolonged resin release by maximizing the effect of the wounding at the injury zone), and 2-chloroethylphosphonic acid (CEPA, an ethylene precursor, signaling molecule involved in stress-induced responses) [5, 6], which are the main active components that elicit the defense response. Although jasmonate is regarded as one of the best resin stimulators because of its capacity to regulate the expression of several genes encoding enzymes of high metabolic flux control in secondary metabolite biosynthesis [7–9], its use is restricted in large-scale operations due to high costs [1]. Several chemical adjuvants have been experimentally validated in the field to improve resin yield and/or to replace the need for the relatively expensive CEPA. Among these are included salicylic acid (mediator in plant responses to pathogens) [10] and auxin (stimulates both ethylene production and the differentiation of resin ducts) [11]. Alternative strategies to modify the composition of oleoresin stimulant paste are based on improving the activity of terpenoid synthases with metal cofactors and activators (e.g., K, Fe, and Mn) or by increasing ethylene sensitivity (adding Cu, a component of ethylene receptor proteins) [1, 3].

Oleoresin yield and quality are objects of interest in pine tree biotechnology, both in terms of environmental and genetic control. Monitoring these parameters in individual trees grown in the field provides a means to examine the control of terpene production in resin canals, as well as the identification of genetic-based differences in resinosis, providing a needed basis for generating super-resinous forests. A detailed protocol on how to prepare different types of stimulant pastes, as well as the basic procedure to tap pine trees using the bark streak technique, is described below.

2 Materials

2.1 Materials for Laboratory Preparation of Resin-Stimulating Pastes

1. Scale.
2. Beakers (500 mL capacity).
3. Graduated cylinder (100 mL capacity).
4. Glass rod.

5. Magnetic stirrer plate and magnetic rod.
6. Plastic squeeze bottles (600 mL capacity).

2.2 Materials for Pine Tapping in the Field

1. Plastic bags.
2. Wire (for belting the plastic bag on the tree).
3. Bark streaking tool or similar device to remove the bark.
4. Protective equipment (goggles, acid-resistant gloves, plastic apron, rubber boots).
5. Metal funnel for resin transfer from filled bags to storage containers.
6. Nylon or metal sieve for removal of debris from resin.

2.3 Reagents and Solutions

1. 98 % (v/v) sulfuric acid.
2. Naphthalene acetic acid.
3. Benzoic acid.
4. Potassium sulfate.
5. CEPA (Ethrel liquid plant growth regulator 240 g/L, Bayer, Calgary, AB, Canada) (*see* **Note 1**).
6. 1M sodium hydroxide (weigh 4 g of sodium hydroxide and prepare a solution of 100 mL with water).
7. Ground rice husk (1 kg of powder).

3 Methods

The four different chemical adjuvants were chosen according to their different modes of action (Table 1).

Usually the squeeze bottle used to apply paste supports about 400–500 mL. All methods herein described consider the total volume of 500 mL. With this volume you can treat approximately 400 trees. It is recommended to prepare fresh batches of resin-stimulating paste for each streaking period of a pine stand (i.e., once every 15 days) to avoid loss of activity by long storage periods.

3.1 Paste Preparation

1. For potassium paste, weigh 43.564 g of potassium sulfate for a 500 mM concentration solution. In a beaker, complete to a volume of 400 mL with water. Add 100 mL of sulfuric acid.
2. With the magnetic stirrer on, add 10 g of sifted rice husk (*see* **Note 2**) at a time until it reaches 200 g in total. Check if the paste sticks to a glass rod without dripping. This is the consistency for efficient stimulatory effect on the fresh wound cuts of the trees.
3. Add the prepared paste into the squeeze bottle (Fig. 1).

Table 1

Mode of action and responses expected for different adjuvants of resin-stimulating pastes evaluated in pine tapping operations (rationale of use)

Elicitor	Mode of action and responses observed
Sulfuric acid	Potential generator of free radicals; increases and prolongs gum resin yields by maximizing the effect of the wounding at the injury zone
Potassium	Activator of conifer terpenoid synthases
Salicylic acid	Mediator of plant responses to pathogens
Benzoic acid	Precursor of salicylic acid
Naphthalene acetic acid	Promotes ethylene biosynthesis (triggers 1-aminocyclopropane-1-carboxylic-acid—ACC—synthase gene transcription) and may induce resin duct formation, as a result of cambial activity stimulation
Copper	Component of ethylene receptors
CEPA	Precursor of ethylene, signaling molecule involved in stress-induced responses



Fig. 1 Squeeze bottles containing different stimulant pastes

4. For naphthalene acetic acid paste, weigh 0.093 g of naphthalene acetic acid for a 1 mM concentration solution. Add a few droplets of 1 M NaOH for helping the dissolution. In a beaker, complete to a volume of 400 mL with water. Add 100 mL of sulfuric acid.
5. With the magnetic stirrer on, add 10 g of sifted rice husk (*see* **Note 2**) at a time until it reaches 200 g in total. Check if the paste sticks to a glass rod without dripping.

6. Add the prepared paste into the squeeze bottle.
7. For benzoic acid paste, weigh 0.610 g of benzoic acid for a 10 mM concentration solution. Add a few droplets of 1 M NaOH for helping the dissolution. In a beaker, complete to a volume of 400 mL with water. Add 100 mL of sulfuric acid.
8. With the magnetic stirrer on, add 10 g of sifted rice husk (*see* **Note 2**) at a time until it reaches 200 g in total. Check if the paste sticks to a glass rod without dripping.
9. Add the prepared paste into the squeeze bottle.
10. For CEPA paste preparation, in a graduated cylinder, add 62.5 mL of Ethrel for a 3 % final concentration solution. Transfer it to a beaker. Complete to a volume of 400 mL of water. Add 100 mL of sulfuric acid.
11. With the magnetic stirrer on, add 10 g of rice husk sifted (*see* **Note 2**) at a time until it reaches 200 g in total. Check if the paste sticks to a glass rod without dripping.
12. Add the prepared paste into the squeeze bottle.

3.2 Tree Tapping

1. In the forest, select the trees for tapping (*see* **Note 3**).
2. In order to facilitate installation of the plastic bag for the first time, you should remove the rough outer bark from the area at the base of the tree, where the plastic bag will be fixed.
3. Belt the plastic bags with a wire around the trunk right above the area you intend tapping to collect the exuded oleoresin (Fig. 2).
4. With a bark shaving tool (Fig. 3), remove a horizontal strip of bark (with circa 2–3 cm in height) across about one-third of the width of the tree, above the plastic bag gutter, to promote resin flow. After bark removal, a clear whitish surface is exposed (cambium and younger sapwood).
5. Apply the stimulant paste on the top portion of the fresh groove formed by bark removal.
6. Remove the bark making horizontal grooves in the tree wood using a bark shaving tool or equivalent instrument every 15 days. The length of the grooves should be kept at about one-third of the tree circumference and the height should remain from 2 to 3 cm.
7. When the plastic bag is full, replace it and fix the new bag as close as possible of the newest groove (Fig. 2).

3.3 Initial Resin Harvest in the Forest

1. Collect the oleoresin accumulated in the bags.
2. If needed, carefully decant any rain water (it will form a separate phase from resin).
3. With a metal funnel, filter oleoresin through nylon or metal sieve to remove debris.



Fig. 2 Aspect of a tapped *Pinus elliottii* tree. The wounded area was formed by periodic streaking of the bark, followed by stimulant paste application. Plastic bags are belted around the trunk to collect the exuded oleoresin

4. Use the filtrate for the desired application (e.g., evaluation of oleoresin mass, terpene composition analyses by GC-MS and/or HPLC-MS, biotransformation reactions, distillation or further fractionation to purify higher value components).

4 Notes

1. CEPA is also known by different commercial names, such as Ethrel and Ethephon.
2. Usually, rice husk comes as large grains or pellets. Rice husk should be sifted through a relatively fine mesh filter to prevent obstruction of the squeeze bottle.

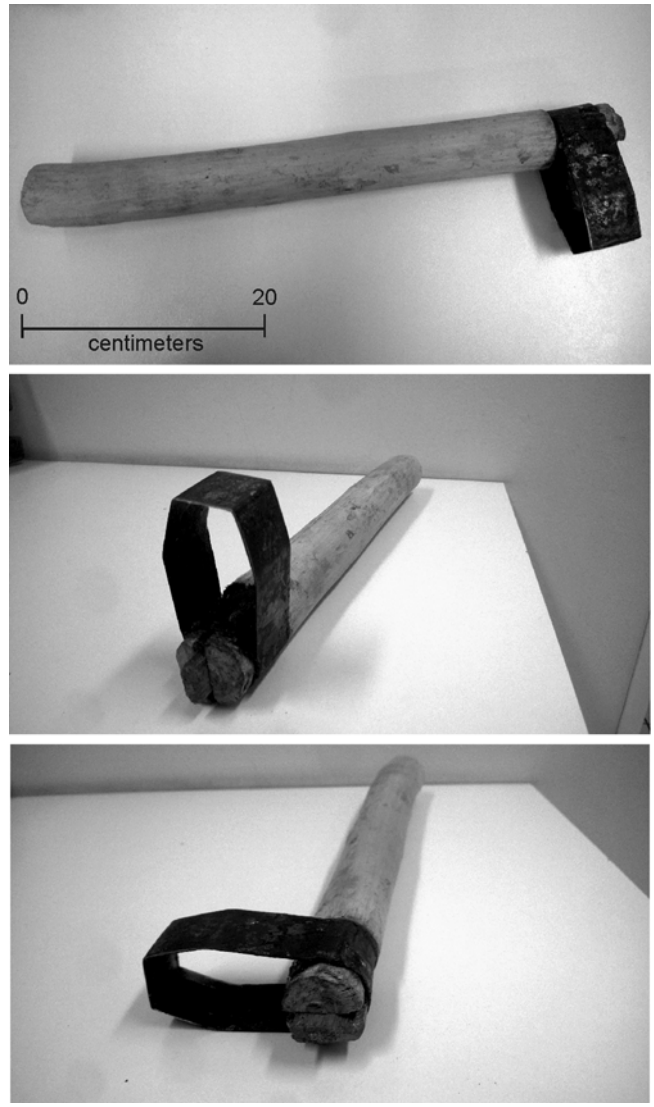


Fig. 3 Views of a bark shaving (streaking) tool

3. To begin, find a mature, live, good-sized, tight-barked pine tree for best results. Choose 10–20-year-old plantations or trees with diameters greater than 20–25 cm to tap. Pine species most suitable for tapping are *Pinus massoniana*, *P. yunnanensis*, *P. elliottii*, *P. caribaea*, *P. merkusii*, *P. kesiya*, *P. oocarpa*, *P. pinaster*, *P. roxburghii*, and *P. tabuliformis*. In most climates, oleoresin flow will be more pronounced in the spring and summer.

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