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Oxidation of monoterpenes in *Protium heptaphyllum* oleoresins

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ABSTRACT

Protium heptaphyllum (Burseraceae) oleoresins are rich in volatile monoterpenes, exhibiting a chemical composition that can be strongly altered with time. The present work aimed to discuss the temporal change of the volatile composition of these oleoresins, and search for related supporting evidence. Samples of *P. heptaphyllum* oleoresin were collected separately for fresh (n = 10) and aged (n = 8) oleoresins, with the essential oils obtained by hydrodistillation analyzed by GC-FID and GC-MS. Fresh oleoresins were characterized by a high content of terpinolene (28.2–69.7%), whereas aged ones contained large amounts of *p*-cymene (18.7–43.0%) and *p*-cymen-8-ol (8.2–31.8%). Multivariate analyses were performed based on the yield and major essential oil components to clearly demonstrate the existence of two subsets (fresh and aged oleoresins). In addition, an analysis of the partial genome sequencing of the species was carried out, producing the largest amount of data for the genus *Protium*. Subsequently, were searched for nucleotide sequences responsible for the enzymes involved in the biosynthesis of monoterpenes. Two hypotheses were formulated to understand the oxidation process during aging of the oleoresins: (i) a natural chemical oxidation of terpenes and (ii) an oxidation catalyzed by enzymes produced by microorganisms associated with the plant. The results suggested that terpinolene was most likely oxidized to *p*-cymene, which, in turn, was oxidized into *p*-cymen-8-ol during natural aging of the exudate due to abiotic factors.

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1. Introduction

Protium heptaphyllum (Aubl.) Marchand belongs to the Burseraceae family of trees, that account for an important part of the structure and diversity of both humid and dry forests in many parts of the tropics (Daly et al., 2012). Trees of *P. heptaphyllum* can reach up to 20 m in height and 60 cm in trunk diameter, often occurring in riparian semi-decidual forests (Lorenzi, 2008). *Protium* species are well known to produce secondary metabolites featuring different types of terpenes, with more than 100 different mono- and sesquiterpenes characterized (Siani et al., 2004; Silva et al., 2009; Marques et al., 2010). In view of this, *Protium* species have been used as models to better understand the natural variation in the genes underlying monoterpene synthesis and the possible drivers of such variation (Zapata and Fine, 2013).

Plants of the *Protium* genus store oleoresins in secretory structures of their bark until injury allows exudation (Langenheim, 2003). The nonvolatile fraction of oleoresins is rich in triterpenoids, e.g., α-amyrin and β-amyrin, while the volatile fraction is composed predominantly of monoterpenes (Rüdiger et al., 2007). Monoterpene production is catalyzed by enzymes encoded by terpene synthase genes that belong to a highly diverse TPS gene family (Bohlmann et al., 1998). The evolutionary history of monoterpene synthases (TPSb) within *Protium* in the context of the TPS family has been assessed by Zapata and Fine (2013), who concluded that *Protium* retained at least three, and possibly up to five, copies

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of TPSb genes, and suggested that this fact is associated with the monoterpene diversity expressed in the genus.

Although *Protium* oleoresins have been studied from genetic, chemical, ecological, and evolutionary aspects, the mechanisms involved in the temporal changes of the produced chemical compounds have not been extensively discussed. The oleoresins of *Protium* can be characterized as fresh or aged, depending on their texture. Fresh oleoresins are known to have a soft, viscous, and malleable nature, which is partially due to the considerable amount of monoterpenes contained therein, which can exceed 20%. About three months after exudation, the oleoresins harden in the bark of the trees, becoming aged and displaying a solid and dry texture. The aged oleoresin can remain in trees for years, and its essential oil content can be reduced to less than 2% (Costa, 1975; Langenheim, 2003; Pontes et al., 2007; Da Silva et al., 2013). Despite the fact that aged oleoresins are mostly used in folk medicine, few studies comparing the composition of essential oils obtained from fresh and aged *Protium* oleoresins have been performed (Siani et al., 1999; Ramos et al., 2000; Pontes et al., 2007).

The aim of the present work was to evaluate the temporal changes in the volatile composition of *P. heptaphyllum* oleoresins. Herein, essential oils from 18 samples of fresh and aged oleoresins were analyzed, and hypotheses explaining the chemical composition changes during aging were discussed.

2. Results

2.1. Chemical compositions and yields of essential oils in fresh and aged P. heptaphyllum oleoresins

The essential oil yields of *P. heptaphyllum* oleoresins ranged from 2.1 to 20.0% (Table 1, Supplementary Material), with best yields obtained for fresh oleoresins (3.4–20.0%) and the lowest ones for aged oleoresins (2.1–6.1%). The average yield of essential oils for fresh oleoresins (12.63 ± 5.05%) was approximately four times greater than that for aged ones (3.24 ± 1.58%).

The chemical compositions of the essential oils of *P. heptaphyllum* oleoresins are given in Table 1 (Supplementary Material), the major components being terpinolene (**1**) (8.8–69.7%), *p*-cymene (**2**) (4.3–43.0%), *p*-cymen-8-ol (**3**) (2.7–31.8%), α-pinene (**4**) (3.6–19.4%), and limonene (**5**) (5.8–11.6%), resembling the chemical profile observed by Lima et al. (2016). α-Terpinene (**6**) (0.8–10.4%) and γ-terpinene (**7**) (0.3–4.6%) were also present in relatively high amounts in the essential oils (Fig. 1). A large quantitative difference in the content of *p*-cymene (**2**), terpinolene (**1**), and *p*-cymen-8-ol (**3**) monoterpenes was observed between the essential oils of fresh and aged oleoresins (Fig. 2). The essential oils obtained from fresh oleoresins ($n = 10$) mainly contained terpinolene (**1**) (28.2–69.7%) and *p*-cymene (**2**) (4.3–23.3%), whereas the ones obtained from aged oleoresins ($n = 8$) mainly contained *p*-cymene (**2**) (18.7–43.0%) and *p*-cymen-8-ol (**3**) (8.2–31.8%), followed by terpinolene (**1**) (8.8–20.9%). Interestingly, the content of *p*-cymene (**2**) is often high in aged essential oils (Hausen et al., 1999; Misharina and Polshkov, 2005; Turek and Stintzing, 2013), with a large reduction of terpinolene (**1**) content recently reported to occur in the essential oils of oregano and laurel after days of storage at 60 °C (Olmedo et al., 2015).

All essential oils comprised monoterpene hydrocarbons (60.8–93.0%), represented mainly by terpinolene (**1**) and *p*-cymene (**2**), and oxygenated monoterpenes (4.8–35.8%), mainly represented by *p*-cymen-8-ol (**3**). Notably, the essential oils of aged oleoresins contained more oxygenated monoterpenes (9.1–35.8%) and consequently less monoterpene hydrocarbons (4.5–12.4%) than those of fresh oleoresins (Table 1). Similarly to the oxygenated monoterpenes, the content of aromatic monoterpenes was also increased in the essential oils of aged oleoresins (46.3–59.6%) compared to those of fresh oleoresins (10.0–33.2%).

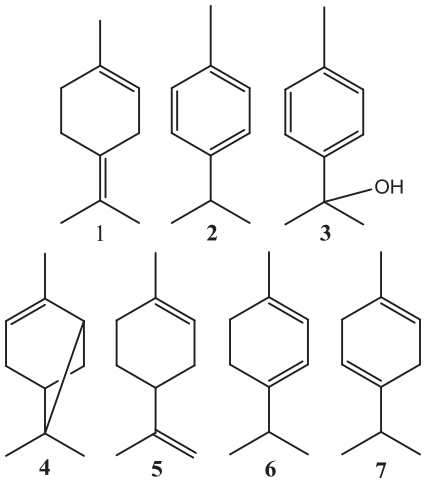

Fig. 1. Major components of *Protium heptaphyllum* oleoresin essential oils.

2.2. Partial genome analysis

A total of 34,332,626 paired-end sequencing reads were produced by partial genome analysis, totaling 8.93 billion of DNA nucleotide bases. The sequencing reads were subjected to a cleaning procedure using Trimmomatic software (Bolger et al., 2014), which discarded most sequences from the R2 dataset due to the presence of sequencing adapters or low quality. The cleaned dataset contained 4,481,402 high-quality paired-end reads and 29,845,377 unpaired reads (mainly from the R1 dataset), totaling 4.74 gigabases of genomic information.

The cleaned reads were used as a query for GMAP alignment against a pre-built catalog of seven enzymes downloaded from the KEGG database, known to account for the biosynthesis of monoterpenes found in other plants. 42 sequencing reads were found with similarities to 14 K15086 (3S)-linalool synthase [EC:4.2.3.25]; 10 reads with similarities to 10 K12467 myrcene/ocimene synthase [EC:4.2.3.15]; 42 reads similar to 2 K12467 β-myrcene/(E)-β-ocimene synthase [EC:4.2.3.15]; 25 reads with similarities to the two paralogs of 1,8-cineole synthase K07385 [EC:4.2.3.108]; and 9 reads with similarities to the two paralogs of a short-chain dehydrogenase/reductase, namely 2 K15095 (+)-neomenthol dehydrogenase [EC:1.1.1.208].

Although the above observation proves that *Protium* shows monoterpene metabolism, it does not clarify whether the conversion of terpinolene (**1**) to *p*-cymene (**2**) and *p*-cymen-8-ol (**3**) is due to its metabolism. Actually, the reported investigations of enzymes responsible for the conversion of terpinolene (**1**) to other compounds are scarce. The KEGG database (KEGG, 2015) presents the most comprehensive catalog of enzymes and metabolic pathways known and contains only enzymes that convert *p*-cymene (**2**) to another compound, named *p*-cumate. This suggests that *p*-cymene (**2**) cannot be converted to *p*-cymen-8-ol (**3**) through the action of any presently known enzyme.

2.3. Chemometric statistical analysis

Prior to multivariate analysis, an analysis of variance (ANOVA) was performed to determine which conditions, if any, influence the yields and monoterpene contents. The collection time and oleoresin type (fresh or aged) were significantly ($p < 0.05$) important for essential oil yields, with the mean values obtained for terpinolene

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