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Research article

Within-plant distribution and emission of sesquiterpenes from *Copaifera officinalis*

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ABSTRACT

Copaifera officinalis, the diesel tree, is known for massive production of oleoresin, mainly composed of sesquiterpene hydrocarbons. In this study, composition of these sesquiterpenes and their concentrations in leaves, stems and roots of *C. officinalis* at two developmental stages, including the three-week old (TW) seedlings and two-year old (TY) trees, were determined. The leaves of TW seedlings and TY trees contained similar number of sesquiterpenes, which also had comparable concentrations. The stems of TW seedlings had higher concentrations of sesquiterpenes than those of TY trees. In contrast, the number of sesquiterpene species and their concentrations in the roots of TW seedlings were much lower than those in the roots of TY trees. Cluster analysis of sesquiterpenes estimated that there are at least four terpene synthase genes involved in the production of sesquiterpenes in *C. officinalis*. Because sesquiterpenes are highly volatile, emissions of sesquiterpenes from healthy and wounded TW seedlings were examined using headspace analysis. Whereas very low emission of sesquiterpenes was detected from undamaged plants, the physically injured seedlings emitted a large number of sesquiterpenes, the quality and the relative quantity of which were similar to those in leaves determined using organic extraction. The implications of our findings to the biosynthetic pathways leading to the production of sesquiterpenes as well as their biological roles in *C. officinalis* are discussed.

1. Introduction

Terpenoids are the largest class of secondary metabolites produced in the plant kingdom and approximately 50,000 of them have been structurally identified [17]. This diverse group of plant metabolites is important for many aspects of plant biology and ecology [23]. For example, some terpenoids are toxic and could function in deterring herbivores [11]. Other terpenoids are produced by flowers as volatiles involved in attracting insect pollinators for plant cross-pollination [20]. Volatile terpenoids can also be emitted from herbivore-damaged plants to attract natural enemies of the feeding herbivores for indirect plant defense [22]. Many terpenoids have potent pharmaceutical activities. For example, taxol [9] and artemisinin [21] are effective medicines for treating cancer and malaria, respectively. Because of their hydrocarbon nature, terpenoids are also attractive as biofuel components; they can be used as combustible fuel in a diesel engine [6]. With a surge of interest in identifying alternative sources of renewable energy, the interest in terpenoid-based biofuel is rekindled [2].

Terpenoids are the main constituents of oleoresins which are produced in high amounts by many tree species. In conifer trees, oleoresin is accumulated within the bark or wood of stems, roots and needles in specialized structures that include resin cells, resin blisters, or reticulate resin duct system [10]. Another well-known example for the massive production of oleoresin is the genus *Copaifera*, which are leguminous trees native to South America. Mature trees can be tapped for an oleoresin called 'copaiba,' which can be directly used in a diesel engine [6]. *Copaifera* species therefore earned the common name "diesel tree." Even though copaiba is an attractive source of biodiesel, its use is currently limited because of the geographical distribution of *Copaifera* trees, which only grow in the tropics. In order to utilize oleoresin as biodiesel, breeding programs might be useful to adapt *Copaifera* to temperate regions. Alternatively, genetic engineering could be used to transfer the entire biochemical pathways and regulatory network for oleoresin production from *Copaifera* into other plants

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to produce novel bioenergy crops suitable for broader geographical regions. It is therefore important to understand how the oleoresin of *Copaifera* trees is synthesized and how its production is regulated at the molecular and biochemical levels. Of the about 70 species within the genus *Copaifera* [14,19], the chemical composition of the oleoresin has been analyzed for a number of species, including *Copaifera guianensis*, *Copaifera duckei*, *Copaifera langsdorfii*, *Copaifera trapezifolia*, *Copaifera cearensis*, *Copaifera reticulata*, and *Copaifera multijuga* [7,12,24,25]. *Copaifera officinalis* is one species that occurs widely throughout South America and can also be found in Puerto Rico and Hawaii. Because of its accessibility, *C. officinalis* was chosen as a model species for the current study.

Plant terpenes are synthesized by the action of terpene synthases (TPSs), which convert geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) to monoterpenes, sesquiterpenes and diterpenes, respectively [23]. Biochemical characterization and molecular phylogenetic analysis of a large number of *TPS* genes from various plant species indicate that this enzyme class shares a common origin [1,3,23]. Several *TPS* genes involved in oleoresin terpenoid production in conifers have been identified and characterized [4,5,16,18]. Unlike conifers, whose oleoresin is mainly composed of monoterpenes and diterpenes, the oleoresin of *Copaifera* trees consists mainly of sesquiterpenes. To elucidate sesquiterpene biosynthesis in *Copaifera*, as a first step, it is important to know where these chemicals are synthesized and accumulated. Whereas mature trees of *Copaifera* contain massive amounts of oleoresin, they are not a good system for studying biosynthesis because of their limited accessibility and difficulty in handling.

In this study we were interested to determine whether the molecular machinery for sesquiterpene production in *Copaifera* trees is functional in young trees. Therefore, our first objective was to determine whether seedlings and young trees of *C. officinalis* produce sesquiterpenes and, if so, to compare their distributions. Understanding the biological functions of terpenoids in *C. officinalis* will also help in the study of their biosynthesis. Oleoresin of *Copaifera* trees has been suggested to play a role in plant defense against pests and pathogens [15]. As aforementioned, many terpenoids function as volatiles. Whether sesquiterpenes in *Copaifera* trees are released from plants, however, is unknown. Thus, the second objective of this study was to determine whether *C. officinalis* plants emit sesquiterpenes and whether the emission can be altered by physical injury. The results might have broad implications toward their biology.

2. Results and discussion

2.1. Distribution of sesquiterpenes in seedlings and two-year old trees

Although the composition of oleoresin produced by a number of species of *Copaifera* has been analyzed, our understanding of accumulation of essential oil in different organs of *Copaifera* trees at different developmental stages is very limited. Extracts of different tissues of three-week old (TW) seedlings of *C. officinalis* were analyzed for sesquiterpene contents (Table 1). The leaf tissue contained 26 sesquiterpenes and the stem tissues contained 25 sesquiterpenes. Two sesquiterpenes were identified in roots. The most abundant sesquiterpene in leaves was germacrene D with a concentration of 322.8 μg/g fresh weight, which accounted for 81.4% of total sesquiterpenes. Germacrene D was also the most abundant sesquiterpene in stems, which had a concentration of 256.2 μg/g fresh weight. Four terpenes, including α-cubebene, cis-α-bergamotene, trans-α-gergamotene and sesquisabinene-B, were only detected in leaves but not in stems. α-gurjunene, β-selinene and α-calacorene were only detected from stems, but not leaves. Twenty-two sesquiterpenes were detected from both leaves and stems. (*E*)-β-caryophyllene was the only compound that was detected from all three organs: leaves, stems, and roots. The other compound detected from roots, α-gurjunene, was also detected from stems. The concentrations of all sesquiterpenes in leaves, stems and roots of young seedlings were 396.3 μg/g fresh weight, 353.15 μg/g fresh weight, and 1.2 μg/g fresh weight, respectively (Fig. 1).

A total of 30 sesquiterpenes was detected from two-year old (TY) trees. The leaf tissue contained a total of 29 sesquiterpenes, the stems tissue contained 25 sesquiterpenes, and the root tissues contained 16 sesquiterpenes (Table 1). The most abundant sesquiterpene in leaves was germacrene D with a concentration of 274.4 μg/g fresh weight, which accounted for 60.7% of total sesquiterpenes. The most abundant sesquiterpene stems was also germacrene D, which had a concentration of 50.8 μg/g fresh weight and composed 68.5% of total sesquiterpenes. The most abundant sesquiterpene in roots was (*E*)-β-caryophyllene, which had a concentration of 164.4 μg/g fresh weight and accounted for 67.7% of total sesquiterpenes in roots. All sesquiterpenes detected in roots were also detected from leaves. In contrast, five terpenes, including α-cubebene, β-bourbonene, cis-α-bergamotene, α-calacorene and selina-3,7-(11)-diene, were detected only in leaves but not stems. α-gurjunene was the only compound from roots that was not detected from leaves. The concentrations of all sesquiterpenes in leaves, stems and roots of TY trees were 451.9 μg/g fresh weight, 74.23 μg/g fresh weight, and 242.95 μg/g fresh weight, respectively (Fig. 1). It was surprising that the concentrations of sesquiterpenes in the stems of TY trees were lower than those in leaves and roots. These data suggest that the high levels of sesquiterpenes in the stems of mature trees might be a result of long-term accumulation, accompanied by the formation of large specialized structures for oleoresin storage.

The species of sesquiterpenes and their relative quantity in TW and TY plants of *C. officinalis* are highly comparable to those of commercial copaiba extracted from mature *C. officinalis* trees (F. Chen, unpublished), indicating that small trees are a good proxy for large trees. In addition, most of the sesquiterpenes that were identified from *C. officinalis* have also been found from oleoresins of other *Copaifera* species [7,12,24,25]. Nevertheless, significant chemical variation occurs among *Copaifera* species. For instance, germacrene D is one of the major components in all tissues that were analyzed in this study and in commercial oleoresin from *C. officinalis* except roots. In contrast, it was not detected or, at least, it was not one of the major compounds in the oleoresins from *C. guianensis*, *C. duckei* and *C. multijuga* [7]. This suggests that although the same biochemical pathway is responsible for the production of oleoresin in *Copaifera*, divergence at the molecular and genetic levels, especially those of sesquiterpene synthases, has occurred among species.

2.2. Emission of sesquiterpenes from intact and wounded seedlings

Terpenoids from *C. officinalis* has been suggested to be important for defense against insects [15]. To understand whether sesquiterpenes are emitted from *C. officinalis*, we analyzed the emission of these sesquiterpenes using a headspace technique. Under normal growing conditions, intact *C. officinalis* plants emitted only trace amounts of terpenoids. When the plants were physically injured, large amounts of terpenoids were detected (Fig. 2). The profile was highly similar to that from leaves of seedlings after organic extraction (Table 2). One major difference was in α-calacorene, which was present in plant tissues but was not detected in the headspace. Wound-caused emission of these

Table 1
Concentrations of individual sesquiterpenes in plant organs.

Compounds	Seedlings			Two-year old trees		
	Leaves	Stems	Roots	Leaves	Stems	Roots
δ-elemene[a]	5.54 ± 0.3[b]	3.19 ± 0.21	ND[c]	5.70 ± 0.11	0.82 ± 0.078	0.28 ± 0.028
α-cubebene	0.20 ± 0.045	ND	ND	0.77 ± 0.011	ND	ND
α-ylangene[a]	0.51 ± 0.0036	1.11 ± 0.076	ND	0.97 ± 0.11	0.32 ± 0.046	22.78 ± 1.8
α-copaene	1.86 ± 0.2	1.60 ± 0.22	ND	4.79 ± 0.30	0.75 ± 0.032	4.15 ± 0.18
7-epi-sesquithujene	0.98 ± 0.053	0.46 ± 0.091	ND	2.23 ± 0.062	0.29 ± 0.023	ND
β-elemene	1.32 ± 0.071	1.47 ± 0.47	ND	4.58 ± 0.26	0.37 ± 0.034	9.44 ± 0.68
α-gurjunene[a]	ND	1.15 ± 0.13	0.21 ± 0.034	ND	ND	16.12 ± 1.1
cis-α-bergamotene	0.10 ± 0.022	ND	ND	0.40 ± 0.10	ND	ND
(*E*)-β-caryophyllene	22.98 ± 1.46	53.35 ± 7.1	1.00 ± 0.040	53.41 ± 7.6	13.29 ± 0.26	164.38 ± 17.7
trans-α-bergamotene	0.70 ± 0.014	ND	ND	ND	ND	ND
Sesquisabinene-A	1.80 ± 0.12	0.97 ± 0.036	ND	3.23 ± 0.011	0.45 ± 0.050	0.31 ± 0.0065
Sesquisabinene-B	0.29 ± 0.030	ND	ND	1.34 ± 0.40	0.09 ± 0.013	ND
4αH,10αH-guaia-1(5),6-diene	0.55 ± 0.064	0.35 ± 0.070	ND	1.26 ± 0.11	0.10 ± 0.012	0.53 ± 0.033
α-humulene	2.94 ± 0.16	6.64 ± 0.39	ND	7.91 ± 0.26	1.45 ± 0.043	13.01 ± 0.86
Allo-aromadendrene	0.60 ± 0.12	0.23 ± 0.099	ND	1.62 ± 0.40	0.13 ± 0.031	0.17 ± 0.013
γ-muurolene	1.23 ± 0.19	1.03 ± 0.013	ND	3.23 ± 0.011	0.36 ± 0.024	ND
α-amorphene[a]	1.50 ± 0.18	1.69 ± 0.28	ND	6.98 ± 0.29	0.69 ± 0.030	3.54 ± 0.23
germacrene D	322.80 ± 19.5	256.18 ± 31.0	ND	274.45 ± 21.5	50.83 ± 0.17	4.11 ± 0.16
β-selinene	ND	3.54 ± 0.36	ND	4.86 ± 0.75	0.54 ± 0.080	ND
Bicyclioesquiphyllandrene	ND	ND	ND	14.14 ± 2.08	0.89 ± 0.0033	1.23 ± 0.077
α-auurolene	7.59 ± 0.54	6.12 ± 0.29	ND	9.60 ± 1.5	0.64 ± 0.015	1.60 ± 0.12
β-bisabolene	8.27 ± 0.17	1.57 ± 0.097	ND	4.87 ± 0.69	0.02 ± 0.0026	0.87 ± 0.065
γ-cadinene	4.27 ± 1.1	4.17 ± 0.61	ND	14.88 ± 3.1	0.80 ± 0.15	0.21 ± 0.026
δ-cadinene	2.88 ± 0.41	3.17 ± 0.13	ND	9.41 ± 0.97	0.53 ± 0.086	ND
cis-calamene	2.93 ± 0.10	1.65 ± 0.18	ND	3.59 ± 0.43	0.18 ± 0.00019	ND
Zonarene	0.73 ± 0.069	0.79 ± 0.013	ND	2.31 ± 0.034	0.03 ± 0.0015	ND
Cakina-1'4-diene	0.94 ± 0.0021	0.24 ± 0.00034	ND	1.72 ± 0.0056	0.1 ± 0.034	ND
α-cadinene	1.31 ± 0.084	0.39 ± 0.13	ND	2.36 ± 0.039	0.11 ± 0.011	ND
α-calacorene	ND	0.45 ± 0.042	ND	2.54 ± 0.051	ND	ND
Selina-3,7(11)-diene	ND	ND	ND	0.78 ± 0.0064	ND	ND
Germacrene B	1.52 ± 0.15	0.70 ± 0.091	ND	2.18 ± 0.056	0.15 ± 0.0051	ND

[a] Compounds whose identity was determined based on commercial database.
[b] μg/g fresh weight. Each value is an average of three independent measurements with two technical replicates.
[c] ND: not detected.

sesquiterpene volatiles might deter natural pests of *C. officinalis* trees. Additionally, they might also act as cues to attract the natural enemies of the herbivores in the context of tritrophic interactions, which has been observed in other systems [13,22,26].

2.3. Cluster analysis of sesquiterpenes from different tissues

We have embarked on isolating and characterizing *TPS* genes that are responsible for production of sesquiterpenes in *C. officinalis*. Before that can be accomplished, however, it is useful to understand the genetic complexity of terpene biosynthesis in *C. officinalis*.

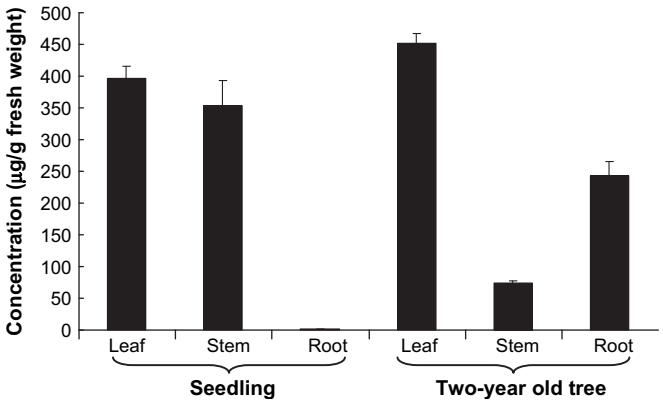

Fig. 1. Concentrations of total sesquiterpene hydrocarbons in different tissues of three-week old seedlings and two-year old young trees of *C. officinalis*.

We were interested in estimating the number of *TPS* genes responsible for production of key sesquiterpenes. Whereas some sesquiterpene synthases form a single product, most of them catalyze the formation of multiple products that appear consistent in relative proportions. Based upon these observations, cluster analysis was performed using concentrations of individual sesquiterpenes across three organs and two developmental stages: leaves, stems and roots of both TW seedlings and TY trees. Four clades were identified which contained 7, 10, 7 and 7 sesquiterpenes respectively (Fig. 3).

Because the compounds in each clade showed similar patterns of accumulation, they are likely the products of a single enzyme. Based on this reasoning, we could predict that four TPS enzymes are involved in the production of sesquiterpenes in *C. officinalis*. The multiple products of a single terpene synthase often share a similar type of cyclization. Each of the clades shows a specific type of cyclization (Fig. 4), supporting the assumption that the products of each clade are formed by one terpene synthase. Some characterized sesquiterpene synthases had similar product profiles as predicted in Fig. 3. For example, the rice (*E*)-β-caryophyllene synthase also produces β-elemene and α-humulene as side products [26], which is the same as predicted in clade 1 (Fig. 3). It should be noted that different sesquiterpene synthases might have overlapping products, i.e., some of the sesquiterpenes in *C. officinalis* are probably produced by more than one TPS enzyme. Making the issue even more complicated, more than one sesquiterpene synthase gene might encode enzymes having similar biochemical functions. Therefore, we estimate that at least four *TPS* genes are responsible for sesquiterpene production in *C. officinalis*, a number which is tractable for genetic manipulation.

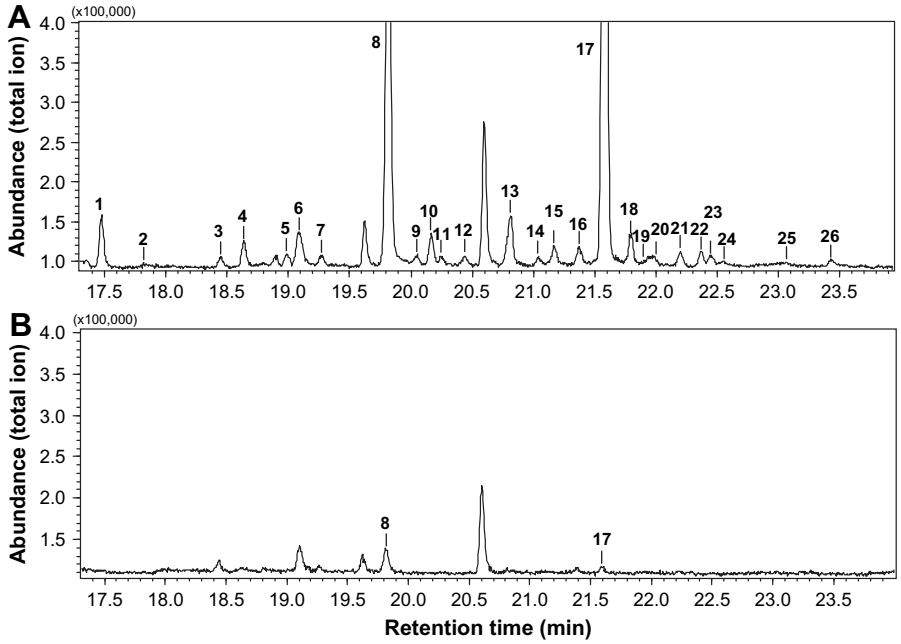

Fig. 2. Emission of sesquiterpene volatiles from the above-ground parts of the physically wounded plants (A) and undamaged plants (B). 1, δ-elemene; 2, α-cubebene; 3, α-ylangene; 4, α-copaene; 5, 7-epi-sesquithujene; 6, β-elemene; 7, α-gurjunene; 8, (*E*)-β-caryophyllene; 9, trans-α-bergamotene; 10, sesquisabinene-A; 11, sesquisabinene-B; 12, 4αH,10αH-guaia-1(5),6-diene; 13, α-humulene; 14, allo-aromadendrene; 15, γ-muurolene; 16, α-amorphene; 17, germacrene D; 18, β-selinene; 19, bicyclosesquiphyllandrene; 20, α-muurolene; 21, β-bisabolene; 22, γ-cadinene; 23, δ-cadinene; 24, cis-calamene; 25, selina-3,7(11)-diene and 26, germacrene B. Unlabelled peaks are not terpenes.

Table 2
Emission of individual sesquiterpenes from wounded plants.

Compounds	Headspace of wounded plants (%)[a]	Organic extraction of leaves (%)
δ-elemene[b]	2.27 ± 0.08	1.40 ± 0.15
α-cubebene	0.23 ± 0.04	0.05 ± 0.014
α-ylangene[b]	0.46 ± 0.02	0.13 ± 0.006
α-copaene	1.18 ± 0.04	0.47 ± 0.074
7-epi-sesquithujene	0.61 ± 0.04	0.25 ± 0.026
β-elemene	1.89 ± 0.43	0.33 ± 0.001
α-gurjunene[b]	0.60 ± 0.08	ND
cis-α-bergamotene	ND[c]	0.03 ± 0.007
(*E*)-β-caryophyllene	21.60 ± 1.45	5.80 ± 0.66
trans-α-bergamotene	0.62 ± 0.13	0.18 ± 0.012
Sesquisabinene-A	1.40 ± 0.06	0.45 ± 0.052
Sesquisabinene-B	0.31 ± 0.12	0.07 ± 0.011
4αH,10αH-guaia-1(5),6-diene	0.26 ± 0.04	0.14 ± 0.009
α-humulene	2.53 ± 0.37	0.74 ± 0.078
Allo-aromadendrene	0.37 ± 0.08	0.15 ± 0.024
γ-muurolene	0.96 ± 0.05	0.31 ± 0.033
α-amorphene[b]	0.89 ± 0.07	0.38 ± 0.027
Germacrene D	59.68 ± 2.04	81.45 ± 0.834
β-selinene	1.65 ± 0.14	ND
Bicyclosesquiphyllandrene	0.14 ± 0.04	ND
α-muurolene	0.36 ± 0.17	1.91 ± 0.04
β-bisabolene	0.53 ± 0.03	2.09 ± 0.15
γ-cadinene	0.70 ± 0.02	1.08 ± 0.23
δ-cadinene	0.54 ± 0.03	0.73 ± 0.068
cis-calamene	0.12 ± 0.01	0.74 ± 0.012
Zonarene	ND	0.19 ± 0.027
Cakina-1'4-diene	ND	0.24 ± 0.011
α-cadinene	ND	0.33 ± 0.004
Selina-3,7(11)-diene	0.08 ± 0.06	ND
Germacrene B	0.34 ± 0.02	0.38 ± 0.02

[a] Relative concentration to the total amount of sesquiterpenes, which was set as 100%. Each value is an average of three independent measurements with two technical replicates.
[b] Compounds whose identity was determined based on commercial database.
[c] ND: not detected.

3. Conclusion

The copious production of oleoresin in *Copaifera* trees makes this genus a good bioenergy crop candidate for the production of biodiesel. The study presented here provides a detailed examination of the developmental regulation of sesquiterpenes, the main constituents of oleoresin in *C. officinalis*. The results indicate that small trees of *C. officinalis* have the full capacity to produce the oleoresin found in the trunk of mature trees. Therefore, the molecular and biochemical mechanisms for production of oleoresin in *C. officinalis* can be studied using small trees as proxies for mature trees. While terpenoids were detected in most organs, it remains unclear exactly where terpenoids are synthesized. They might be just stored in certain organs following transport from another part of the plant. Nevertheless, the rapid accumulation of high levels of sesquiterpenes in leaves and stems of TW seedlings suggests that the molecular machinery for terpene production is functional in those two organs. Although the chemical composition of sesquiterpenes is complex, the genetic basis of their production could be relatively simple, as revealed by the cluster analysis (Fig. 3). Four *TPS* genes were predicted to be mainly responsible for the production of the complex mixture of sesquiterpenes in *C. officinalis*. A large number of *TPS* genes have been isolated from various plants species [23]. Some of these genes were isolated based on the correlation between terpene production and expression of candidate genes [8,26]. Such an approach could be useful for isolating *TPS* genes from *C. officinalis*. Obtaining information about candidate *TPS* genes and their expression patterns from *C. officinalis* will be facilitated using genomic tools, such as pyrosequencing techniques.

4. Materials and methods

4.1. Plants and chemicals

C. officinalis seeds from a single parent were obtained from James Ackerman at the University of Puerto Rico. Seeds were

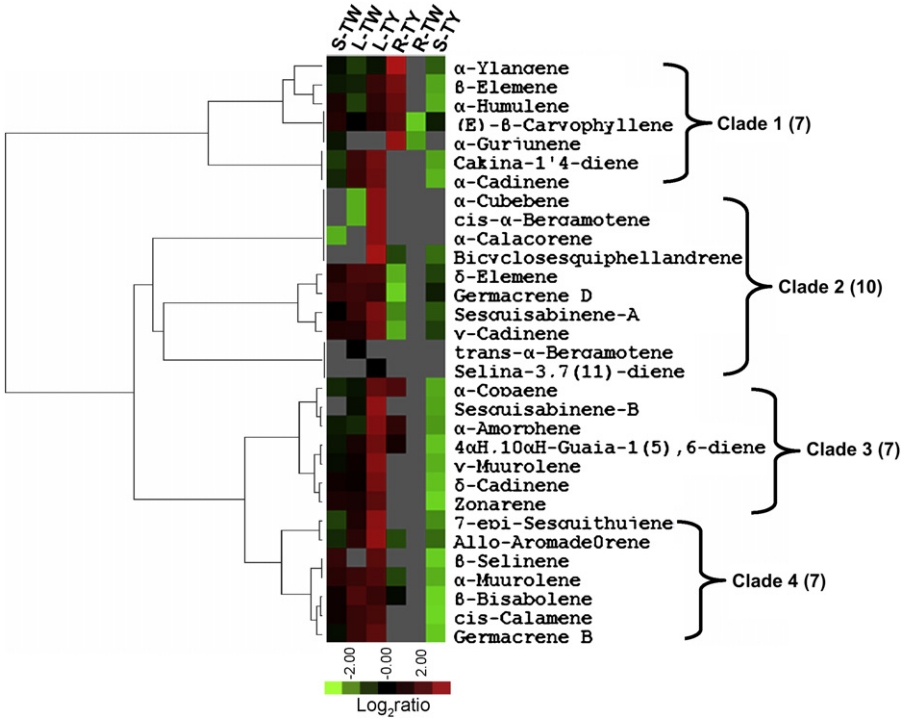

Fig. 3. Cluster analysis of sesquiterpenes. Hierarchical K-means cluster analysis was performed based on chemicals from six tissues. S, L and R represent stems, leaves and roots respectively. Sesquiterpenes were grouped in four clades (clade 1–4). The numbers in parenthesis denote the number of compounds in each clade.

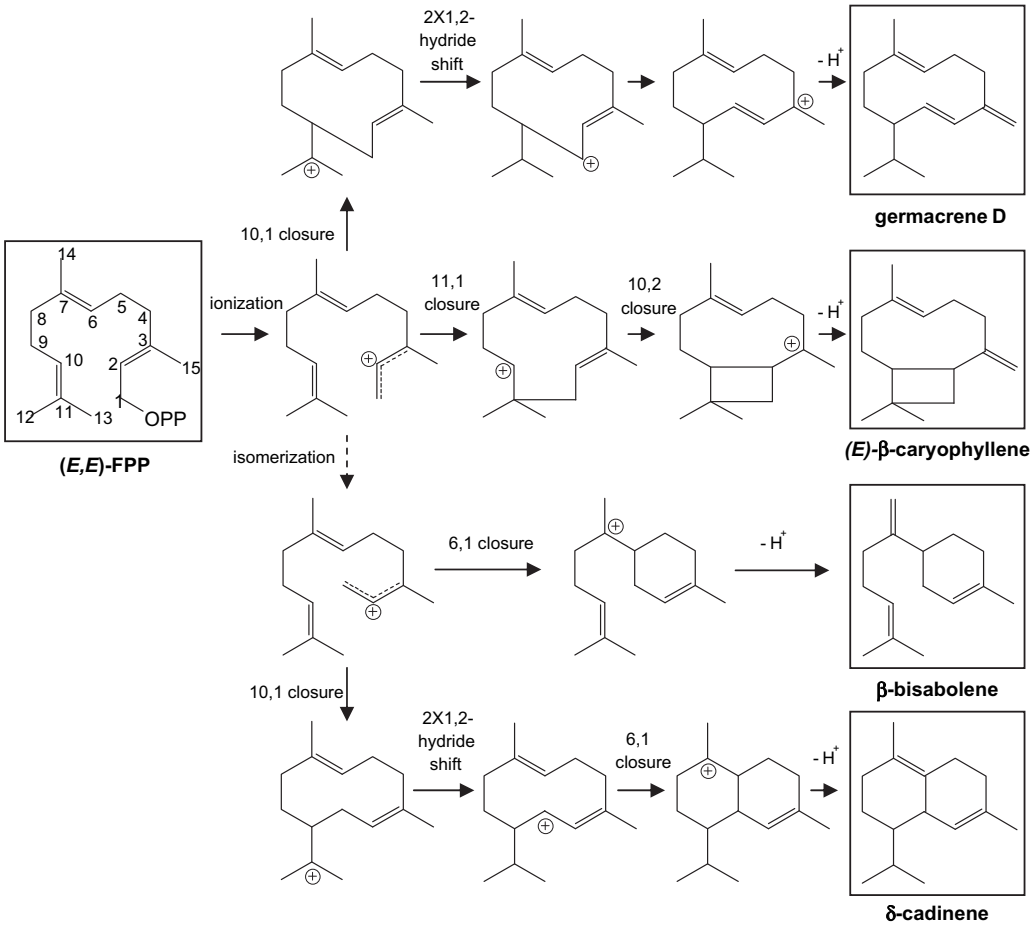

Fig. 4. Schematic mechanisms for biosynthesis of the representative major sesquiterpenes in each of the four clades identified in Fig. 3, germacrene D, (*E*)-β-caryophyllene, β-bisabolene, and δ-cadinene, from their common precursor (*E,E*)-FPP.

surface sterilized in 10% household bleach (Clorox, 6.15% sodium hypochlorite) for 5 min and then moved to 70% ethanol for an additional 5 min. Then seeds were washed three times with sterile water and planted in soil. Seedlings started to emerge after 20 days at 28 °C in 16 h of light and 8 h of darkness. Seedlings were transferred to greenhouse and grown in one part Turface MVP ceramic soil (Profile) conditioner to two parts 3B Mix Professional Formula (Fafard) fertilized with Osmocote 20-20-20 slow release fertilizer (Jacks Classic). The general condition for maintenance of two-year old (TY) trees was 16/8 light/dark photoperiods at 32 °C. Leaves, stems and roots were separated from three-week old seedlings (after emergence) and two-year old trees. For authentic terpene standards, α-copaene, (*E*)-β-caryophyllene and α-humulene were obtained from Sigma–Aldrich (St, Louis, MO, USA), α-cubebene and allo-aromadendrene were ordered from Fluka (Seelze, Germany), all other available terpene standards were kindly provided by W. König (Hamburg, Germany). All other chemicals were purchased from Sigma–Aldrich (St, Louis, MO, USA) unless stated otherwise.

4.2. Organic extraction

Plant tissues were frozen in liquid nitrogen and ground into powder. Ethyl acetate was added (1 ml per 0.2 g tissue), and 1-octanal was added (0.003% w/v) as an internal standard. Extraction proceeded for 2 h at room temperature with continuous shaking. Two microliters of extract was injected into a GC–MS for separation and identification of the terpene compounds.

4.3. Headspace collection

Volatiles emitted from the aerial parts of mechanically wounded TW seedlings and undamaged control plants of *C. officinalis* were collected in an open headspace sampling system (Analytical Research Systems, Gainesville, FL, USA). For physical wounding, leaves were cut with a sterile razor blade to produce two lateral incisions on each side of the midvein. Intact and wounded seedlings were placed in single flasks with about 150 ml distilled water and held in 10 cm (3.9 in) diameter by 30 cm (11.8 in) tall glass chambers that included a removable O-ring snap lid with an air outlet port. Charcoal-purified air entered the chamber at a flow rate of 0.8 L/min from the top through a Teflon hose. Volatiles were collected for 4 h by pumping air from the chamber through a SuperQ volatile collection trap (Analytical Research Systems, Gainesville, FL, USA) and eluted using 100 μL methylene chloride containing 1-octanal (0.003% w/v) as an internal standard.

4.4. GC–MS analysis

Terpenoids from organic extractions and headspace collections were analyzed on a Shimadzu 17A gas chromatograph coupled to a Shimadzu QP5050A quadrupole mass selective detector. Separation was performed on a Restek SHR5XLB column (30 m × 0.25 mm i.d. × 0.25 μm thickness) under the following conditions: helium was the carrier gas (flow rate of 5 ml min^{-1}), a splitless injection (injection injector temperature- 250 °C) was used, and a temperature gradient of 5 °C/min from 40 °C (3-min hold) to 240 °C was applied. Products were identified using the National Institute of Standards and Technology (NIST) mass spectral database and by comparison of retention times and mass spectra with authentic reference compounds if available. Quantification was performed as previously reported [8]. Representative single-ion peaks of each compound were integrated and compared with the equivalent response of the internal standard (single-ion method).

4.5. Hierarchical clustering and data visualization

The concentrations of individual sesquiterpenes in each organ were converted to percentages of the total amount of sesquiterpenes present. These values were used in hierarchical cluster analysis, which was conducted using Cluster 3.0 software (Stanford University, Stanford Microarray Database) using average linkage analysis. Heat maps were created using the Java TreeView 1.60 software.

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References

[1] S. Aubourg, A. Lecharny, J. Bohlmann, Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*, Mol. Genet. Genomics 267 (2002) 730–745.
[2] J. Bohlmann, C.I. Keeling, Terpenoid biomaterials, Plant J. 54 (2008) 656–669.
[3] J. Bohlmann, G. Meyer-Gauen, R. Croteau, Plant terpenoid synthases: molecular biology and phylogenetic analysis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 4126–4133.
[4] J. Bohlmann, M. Phillips, V. Ramachandiran, S. Katoh, R. Croteau, cDNA cloning, characterization, and functional expression of four new monoterpene synthases of the TPSd gene family from grand fir (*Abies grandis*, Arch. Biochem. Biophys. 368 (1999) 232–243.
[5] J. Bohlmann, C.L. Steele, R. Croteau, Monoterpene synthases from grand fir (*Abies grandis*): cDNA isolation, characterization and functional expression of myrcene synthase, (−)-(4S)-limonene synthase, and (−)-(1S:5S)-pinene synthase, J. Biol. Chem. 272 (1997) 21784–21792.
[6] M. Calvin, Hydrocarbons from plants: analytical methods and observations, Naturwissenschaften 67 (1980) 525–533.
[7] V. Cascon, B. Gilbert, Characterization of the chemical composition of oleoresins of *Copaifera guianensis* Desf., *Copaifera duckei* Dwyer and *Copaifera multijuga* hayne, Phytochemistry 55 (2000) 773–778.
[8] F. Chen, D. Tholl, J.C. D'Auria, A. Farooq, E. Pichersky, J. Gershenzon, Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers, Plant Cell. 15 (2003) 481–494.
[9] R. Croteau, R.E.B. Ketchum, R.M. Long, R. Kaspera, M.R. Wildung, Taxol biosynthesis and molecular genetics, Phytochem. Rev. 5 (2006) 75–97.
[10] A. Fahn, Secretory Tissues in Plants, Academic Press, New York, 1979.
[11] J. Gershenzon, R. Croteau, Terpenoids, in: G.A. Rosenthal, M.R. Berenbaum (Eds.), Herbivores. Their Interactions with Secondary Plant Metabolites, The Chemical Participants, vol. 1, Academic Press, New York, 1991, pp. 165–219.
[12] N.V. Gramosa, E.R. Silveira, Volatile constituents of *Copaifera langsdorffii* from the Brazilian northeast, J. Essent. Oil Res. 17 (2005) 130–132.
[13] T.G. Köllner, M. Held, C. Lenk, I. Hiltpold, T.C.J. Turlings, J. Gershenzon, J. Degenhardt, A maize (*E*)-beta-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties, Plant Cell. 20 (2008) 482–494.
[14] J.H. Langenheim, Leguminous resin-producing trees in Africa and South America, in: B.J. Meggers, E.S. Ayensu, W.D. Duckworth (Eds.), Tropical Forest Ecosystems in Africa and South America: a Comparative Review, Smithsonian Institution Press, Washington, DC, 1973, pp. 89–104.
[15] J.H. Langenheim, C.L. Convis, C.A. Macedo, W.H. Stubblebine, *Hymenaea* and *Copaifera* leaf sesquiterpenes in relation to lepidopteran herbivory, Biochem. Syst. Ecol. 14 (1986) 41–49.
[16] D.M. Martin, J. Fäldt, J. Bohlmann, Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily, Plant Physiol. 135 (2004) 1908–1927.
[17] D. McCaskill, R. Croteau, Prospects for the bioengineering of isoprenoid biosynthesis, Adv. Biochem. Eng. Biotechnol. 55 (1997) 107–146.
[18] M.A. Phillips, M.R. Wildung, D.C. Williams, D.C. Hyatt, R. Croteau, cDNA isolation, functional expression, and characterization of a (+)-alpha-pinene synthase and (−)-alpha-pinene synthase from loblolly pine (*Pinus taeda*): stereocontrol in pinene biosynthesis, Arch. Biochem. Biophys. 411 (2003) 267–276.
[19] C. Plowden, Production ecology of Copaiba (*Copaifera* spp.) oleoresin in the eastern Brazilian Amazon, Econ. Bot. 57 (2003) 491–501.
[20] R.A. Raguso, E. Pichersky, New perspectives in pollination biology: floral fragrances. A day in the life of a linalool molecule: chemical communication in

a plant-pollinator system. Part 1: linalool biosynthesis in flowering plants, Plant Species Biol. 14 (1999) 95–120.
[21] R. Sarpong, J.D. Keasling, Production of the antimalarial drug precursor artemisinic acid in engineered yeast, Nature 440 (2006) 940–943.
[22] C. Schnee, T.G. Kollner, M. Held, T.C. Turlings, J. Gershenzon, J. Degenhardt, The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 1129–1134.
[23] D. Tholl, Terpene synthases and the regulation, diversity and biological roles of terpene metabolism, Curr. Opin. Plant Biol. 9 (2006) 297–304.
[24] V.F. Veiga, A.C. Pinto, H.C. de Lima, The essential oil composition of *Copaifera trapezifolia* hayne leaves, J. Essent. Oil Res. 18 (2006) 430–431.
[25] V.F. Veiga Junior, E.C. Rosas, M.V. Carvalho, M.G.M.O. Henriques, A.C. Pinto, Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* hayne – a comparative study, J. Ethnopharmacol. 112 (2007) 248–254.
[26] J.S. Yuan, T.G. Köllner, G. Wiggins, J. Grant, J. Degenhardt, F. Chen, Molecular and genomic basis of volatile-mediated indirect defense against insects in rice, Plant J. 55 (2008) 491–503.