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Differences in Monoterpene Biosynthesis and Accumulation in *Pistacia palaestina* Leaves and Aphid-Induced Galls

Karin Rand[1,2] · Einat Bar[2] · Matan Ben Ari[1] · Rachel Davidovich-Rikanati[2] · Natalia Dudareva[3] · Moshe Inbar[1] · Efraim Lewinsohn[2]

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Abstract Certain insect species can induce gall formation on numerous plants species. Although the mechanism of gall development is largely unknown, it is clear that insects manipulate their hosts' anatomy, physiology, and chemistry for their own benefit. It is well known that insect-induced galls often contain vast amounts of plant defensive compounds as compared to non-colonized tissues, but it is not clear if defensive compounds can be produced *in situ* in the galled tissues. To answer this question, we analyzed terpene accumulation patterns and possible independent biosynthetic potential of galls induced by the aphid *Baizongia pistaciae* L. on the terminal buds of *Pistacia palaestina* Boiss. We compared monoterpene levels and monoterpene synthase enzyme activity in galls and healthy leaves from individual trees growing in a natural setting. At all developmental stages, monoterpene content and monoterpene synthase activity were consistently (up to 10 fold on a fresh weight basis) higher in galls than in intact non-colonized leaves. A remarkable tree to tree variation in the products produced *in vitro* from the substrate geranyl diphosphate by soluble protein extracts derived from individual trees was observed. Furthermore, galls and leaves from the same trees displayed enhanced and often distinct biosynthetic capabilities. Our results clearly indicate that galls possess independent metabolic capacities to produce and accumulate monoterpenes as compared to leaves. Our study indicates that galling aphids manipulate the enzymatic machinery of their host plant, intensifying their own defenses against natural enemies.

Keywords Defensive compounds · Host manipulation · Insect galls · Monoterpene synthase · Monoterpenes

Karin Rand and Einat Bar equally contributed to this work.

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✉ Efraim Lewinsohn
twefraim@agri.gov.il

[1] Department of Evolutionary & Environmental Biology, University of Haifa, Mount Carmel, 31905 Haifa, Israel

[2] Newe Ya'ar Research Center, Agricultural Research Organization, Institute of Plant Sciences, 30095 Ramat Yishay, Israel

[3] Department of Biochemistry, Purdue University, West Lafayette, IN 47907-1165, USA

Introduction

Galls are modified plant structures, the formation of which is induced by other organisms such as bacteria, viruses, and insects. Insects that induce gall development often obtain easy food access and gain protection from harsh environmental conditions and natural enemies (Price et al. 1987). The galling habit has independently evolved numerous times within and among insect lineages indicating that galling is a highly adaptive and evolutionary advantageous phenomenon (Shorthouse and Rohfritsch 1992). Still, little is known about the mechanism of gall formation and maintenance and the way insects recruit plant biochemical, physiological and developmental pathways for their own benefit (Giron et al. 2016; Nabity et al. 2013).

Because galls are often long-lasting and conspicuous, the insects within may be vulnerable to a large variety parasitoids and predators. Furthermore, the closed cavities may promote pathogenic infections (Abrahamson and Weis 1997; Gerchman and Inbar 2011; Inbar et al. 2010). Galls defense mechanisms are dependent on plant-derived physical and chemical traits such as thickness, toughness, nectaries and

customized modified chemical content (Hartley 1998; Nyman and Julkunen-Tiitto 2000; Stone and Schönrogge 2003). Insect-derived galls often contain substantially higher levels of metabolites, such as phenolics and terpenes (Allison and Schultz 2005; Caputo et al. 1979; Cornell 1983; Hartley 1998; Inbar et al. 2003; Koyama et al. 2004; Nyman and Julkunen-Tiitto 2000; Price et al. 1987; Rand et al. 2014) as compared to non-colonized leaves and stems, although the actual efficiency of such compounds to serve as protectants has rarely been tested (see Rostás et al. 2013).

Here we focus on the terpenoid metabolism of galls induced by the aphid *Baizongia pistaciae,* on *Pistacia palaestina* and *P. terebinthus* (the European sibling species of *P. palaestina*) trees in the Mediterranean maquis in Israel. In spring, aphids induce conspicuous, banana-like galls (Ben-Shlomo and Inbar 2012; Kurzfeld-Zexer et al. 2010; Wool 2012). The incipient galls develop on the leaflets, but eventually take over the entire apex of the branch. Each gall may support thousands of phloem-feeding aphids for nearly eight months (spring-fall). The galls act as strong sinks for phloem sap, often originating in distant tissues away from the galls (Burstein et al. 1994; Inbar et al. 2004; Wool 2012). The galls of *B. pistaciae* like other related aphid species (Fordini) that induce galls on wild *Pistacia* trees, contain high levels of tannins and terpenes. The terpenoid fraction consists of complex mixtures of volatile monoterpenes and sesquiterpenes, as well as nonvolatile triterpene compounds (Caputo et al. 1979; Rand et al. 2014; Rostás et al. 2013). *Pistacia* and other Anacardiaceae possess interconnected systems of oil or resin ducts containing terpenoid material (Joel and Fahn 1980; Langenheim 1990) and it could be that the terpenes accumulated in gall tissues are transported there from adjacent or distant tissues to galls. Several lines of evidence indicate that galls possess an independent ability to produce and accumulate monoterpenes. Previous reports had indicated that *B. pistaciae*-induced galls display a markedly different terpene composition than non-colonized leaves (Flamini et al. 2004). Furthermore, we have recently shown that these galls accumulate significantly higher levels of volatile monoterpenes than intact leaves from the same tree, while leaves generally accumulate higher levels of sesquiterpenes than galls (Rand et al. 2014). We also found, that gall terpene compositions differ from those of the supporting trees, and there are remarkable variations in terpene quantity and compositions among trees (Rand et al. 2014). The differences in the terpenoid levels and compositional profiles in galls as compared to intact non-colonized leaves could result either from the differential mobilization, metabolism, and evaporation of metabolites synthesized in leaves and transported to galls or by the action of distinct enzymatic activities present in galls.

Terpenes are the most structurally diverse class of plant metabolites (Croteau et al. 2000; Gershenzon and Croteau 1991; Keeling and Bohlmann 2006). They play important roles as pollinator attractants, growth regulators, and stabilizers of membrane structure as well as in mediating direct and indirect plant defenses (Erbilgin et al. 2006; Heil 2014; Kessler and Baldwin 2001; Wittstock and Gershenzon 2002). Monoterpene synthases are key enzymes responsible for monoterpene formation in plants and have been characterized in conifers and in numerous angiosperm species (Bohlmann et al. 1998, Chen et al. 2011). Most monoterpene synthases utilize geranyl diphosphate (GPP) as a substrate to produce either single or multiple monoterpene products (Falara et al. 2011). Terpene synthase activity has not been previously reported in *Pistacia* or in any other member of the Anacardiaceae, although the expression of genes putatively encoding monoterpene synthases and other enzymes in the terpenoid pathway has been described in developing fruits of mango (*Magnifera indica* L.), a member of the Anacardiaceae family (Pandit et al. 2010).

The main aim of this research was to determine whether gall tissues possess monoterpene biosynthetic capacities autonomous to those that might be present in leaves. Specifically, we asked if monoterpene synthases are active in insect-induced galls and what are their levels in such tissues relative to non-colonized leaves. We also quantified the monoterpene levels and monoterpene synthase activity over the course of leaf and gall development. We show here that despite the high chemical and biochemical variation found among individual trees, the array of monoterpenes produced by monoterpene synthase activities extracted from gall tissues is often substantially different from that of intact leaves. These data support the existence of independent biochemical capacities for monoterpene biosynthesis in insect induced galls as compared to non-colonized leaf tissues.

Methods and Materials

Collection of Leaves and Galls for Volatile Accumulation and Enzymatic Analysis We marked and sampled eighteen naturally growing *P. palaestina* trees on Mount Carmel and the Yezreel Valley (Northern Israel). Six trees were sampled for volatiles during the spring, summer and autumn of 2010. Twelve additional trees were sampled for monoterpene synthase enzyme activity during the spring, summer, and autumn of 2012. At least three leaves and three galls from each tree were collected at three developmental stages as shown in Fig. S1. Leaves: Young unfolded (March) (Stage A). Pre mature fully opened (April) (Stage B). Old leaves before senescence (October) (Stage C). Galls (Bottom panels): Young small (5–20 mm) containing the fundatrix and few offspring (May) (Stage A). Pre mature, developing galls (mm 15–25) containing 10–100 aphids (June) (Stage B). Fully mature galls before they open (October) (Stage C). The galls were cut open, and the aphids were carefully removed with a fine brush

and gently washed with acetone. The samples were then fast frozen with liquid nitrogen and stored at −80^0 until analysis.

Identification and Quantification of Volatile Terpenes in Galls and Leaves We ground 0.2 g of frozen leaf or gall tissue in the presence of liquid nitrogen, and extracted the powder with 2 mL of methyl *tert*-butyl ether (MTBE) containing 25 PPM of ethyl myristate as an internal standard by vigorous shaking overnight at room temperature (Rand et al. 2014). The ethereal phase was separated, dried with anhydrous Na_2SO_4, and analyzed by gas chromatography coupled with mass spectroscopy (GC/MS) (Agilent Technologies (http://www.home.agilent.com/agilent/home.jspx?cc=US&lc=eng).

An aliquot of 1 μl of the concentrated MTBE extract was injected into an Agilent GC/MS system (model 6890 N/5973 N Agilent Technologies CA, USA) equipped with an Rxi-5sil MS column (30-m long × 0.25-mm inner diam, 0.25-μm film thickness and stationary phase 95% dimethyl–5% diphenyl polysiloxane, Restek). Helium (0.8 ml min^{-1}) was used as a carrier gas with splitless injection. The injector temperature was 250 °C, and the detector temperature was 280 °C. The following conditions were used: initial temperature 40 °C for 5 min followed by a ramp from 40 to 120 °C at a rate of 5 °C min^{-1} followed by a ramp from 120 to 280 °C at a rate of 25 °C min^{-1}. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41 to 350 *m/z*. The volatiles were identified by comparison of their relative retention indices and mass spectra with those of authentic samples or with those found in the literature and supplemented with NIST 98 and QuadLib 2205 GC/−MS libraries. A mixture of straight-chain alkanes (C_7-C_{23}) was injected into the column under the above-mentioned conditions for retention indices calculation. Amounts of target components in each sample were calculated as (peak area x internal standard response factor) divided by (response factor x internal standard peak area) as described in Rand et al. (2014).

Measurement of Monoterpene Synthase Activity Monoterpene synthase activity was measured by two complementary methods as indicated. The radioactivity-based method is easy and quantitative but measures the total monoterpene synthase activity levels (see below). A GC/MS based method enables the separation and identification of the products generated.

Extraction of Soluble Protein for the Measurement of Monoterpene Synthase Activity We ground 0.3 g leaf or gall tissues in the presence of liquid N_2, 0.1 g sea sand and 0.1 g polyvinyl polypyrrolidone (PVPP) to adsorb phenolic materials (Lewinsohn et al., 1991a, b). The crushed powder was transferred into 1 ml chilled buffer (50 mM BisTris (pH 7.1), 5 mM ascorbic acid, 5 mM $Na_2S_2O_5$, 5 mM dithiothreitol, 10% (*v*/*v*) glycerol, 10 μM $MnCl_2$ 10 mM $MgCl_2$, and 10% (*w*/*v*) polyvinylpyrrolidone PVP-40). The tubes were centrifuged at 15,000 *g* for 2 min at 4 C. The supernatant collected into a clean chilled vial.

Total Monoterpene Synthase Activity Assay Using Radiolabeled Geranyl Diphosphate as a Substrate Total monoterpene synthase enzymatic assays were achieved by adding crude soluble protein (50 μl soluble protein) into reaction tubes containing 1 ml *n*-hexane with total volume of 100 μl buffer (50 mM BisTris (pH 7.1), 5 mM ascorbic acid, 5 mM $Na_2S_2O_5$, 5 mM dithiothreitol, 10% (*v*/v) glycerol) 10 μM $MnCl_2$, and 10 mM $MgCl_2$ (Lewinsohn et al. 1991a, b). The substrate, ^3H–GPP, specific activity 20 Ci/mmol (ARC), was mixed with 10 μM unlabeled GPP. The vials were vigorously mixed (vortex) and centrifuged for 30 s. The tubes were incubated for 2 h at 30 °C. Upon completion of the reaction, the tubes were mixed again and centrifuged for 1 min. A 650 μl aliquot of the *n*-hexane fraction was treated with about 30 mg Silica Gel 60 A to adsorb alcohols. Subsequently, 500 μl of the mixture were added to 3 ml scintillation liquid Ultima Gold (Perkin Elmer), and radioactivity was determined using a liquid scintillation Analyzer Tri-Carb 2800TR (Perkin Elmer precisely http://www.perkinelmer.com/) counter. Crude protein (50 μl soluble protein) were boiled for 5 min and used as controls. Product amounts were calculated on the basis of the specific activity of the substrate and the counting efficiency of the machine (Gonda et al. 2010; Lewinsohn et al. 1991a, b).

Purification of Soluble Protein Extracts by DE52 Anion Exchange Chromatography and Measurement of Monoterpene Synthase Activity Composition by GC/MS Frozen galls or leaves (both at Stage A, 0.6 g) were crushed with a chilled mortar and pestle in the presence of liquid N_2, 0.1 g sea sand and 0.1 g PVPP. The crushed powder was transferred to 2 ml chilled buffer (50 mM BisTris (pH 7.1), 5 mM ascorbic acid, 5 mM $Na_2S_2O_5$, 5 mM DTT, 10% (*v*/*v*) glycerol, and 1% (*w*/*v*) PVP-40), filtered through Miracloth, and centrifuged at 15,000 *g* for 2 min at 4 °C. The extracts were loaded into 2 ml of DE52 (diethylaminoethyl cellulose) nion exchanger (Whatman) column that was first sequentially washed and equillibratedd with 5 ml of 1 M NaCl, 5 ml DDW, 5 ml half strength buffer without PVP-40. One ml of the soluble extracted protein was loaded onto the DE52 column and eluted sequentially with half strength buffer alone, 100 mM NaCl, 250 mM NaCl, and 500 mM NaCl, fractions of 0.5 ml were collected. The monoterpene synthase activity eluted at the 250 mM NaCl fraction. Purified protein extracts 50 μl, 10 μM geranyl diphosphate (GPP), 10 μM $MnCl_2$ and 10 mM $MgCl_2$, in a total volume of 400 μl were mixed in Teflon capped 2 ml glass GC vials. The vials were incubated overnight at 30 °C and analyzed by solid phase micro extraction (SPME). A 57298-U SPME fiber assembly

Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, Supelco) needle size 23 ga, StableFlex, was used with an autosampler and analyzed by GC/MS as described above. The same GC/MS conditions were used for the identification of monoterpenes as described above in the "*Identification and Quantification of Volatile Terpenes in Galls and Leaves*" section.

Statistical Analyses A Two-Way repeated measurement ANOVA test was used to check for differences between the different groups, with developmental stages as one factor, and tissue type (galls vs. leaves) as a second factor for monoterpenes accumulation and production between three developmental stages and between galls and leaves. Paired-samples *t*-test was used to analyze the differences in monoterpene synthase enzyme activity between galls and leaves within the same tree. Monoterpene synthase enzyme activity in galls and leaves were normally distributed. Data was analyzed using SPSS statistics 17.0 software.

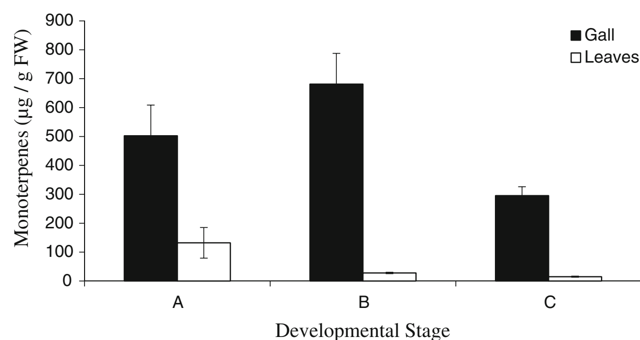


Fig. 1 Monoterpene accumulation in leaves of *Pistacia palaestina* and *Baizongia pistaciae* induced galls at three developmental stages. Leaves: *Stage A* - Young unfolded (March). *Stage B* - Pre mature fully opened (April). *Stage C* - Old leaves before senescence (October). Galls: *Stage A* - Young small (5–20 mm) galls containing the fundatrix and few offspring (May). *Stage B* - Pre mature, developing galls (mm 15–25) containing 10–100 aphids (June). *Stage C* - Fully mature galls before they open (October). Averages ± SE of 6 determinations from individual trees are shown. At all developmental stages, galls' monoterpene levels (*black column*) are significantly higher than leaf monoterpenes (*white columns*) ($F_{2,10} = 8.894$, $P < 0.05$)

Results

Accumulation of Monoterpenes in Galls and Intact Non-colonized Leaves During Development Metabolic profiling revealed that galls accumulate significantly higher levels of volatile monoterpenes than leaves at all developmental stages with up to 24-fold higher level at developmental stage B (Fig. 1). The interaction between the factors stage x tissue (gall/leaf) was significant ($F_{2,10} = 8.894$, $P < 0.05$), indicating that differences between galls and leaves change according to the developmental stage. Monoterpene levels per g fresh weight (FW) in galls remained constantly high at all the three analyzed stages concomitantly with an increase in the gall weight and size during development. In contrast, young intact leaves (stage A) accumulated higher levels of monoterpenes than later developmental stages, in which monoterpene levels gradually decreased albeit did not completely diminish (Fig. 1).

Total Monoterpene Synthase Activity in Galls and Non-Colonized Leaves During Development

Differential Regulation Patterns of Monoterpene Synthase Activity in Leaves and Galls To biochemically rationalize the monoterpene accumulation patterns and determine whether gall tissues possess independent abilities to generate monoterpenes, we measured the total monoterpene synthase activity in soluble protein extracts derived from galls and non-colonized leaves at different developmental stages.

Galls possessed enhanced monoterpene biosynthetic capacity at all stages during gall development as compared to non-colonized leaves that were sampled from the same tree (Fig. 2). The interaction between the two factors was significant $F_{1,2} = 41.804$ $P < 0.05$. In contrast to galls, monoterpene synthase activity in leaves was higher in the beginning of the development (stage A) and then slightly decreased at stages B and C (Fig. 2). However, the mature leaves still have a substantial ability to synthesize monoterpenes (Fig. 2), and the total monoterpene levels in mature leaves is still prominent, albeit at much lower levels than in young tissues (Fig. 1). Overall, averaging among the three developmental stages, galls displayed about 10 fold higher monoterpene synthase enzyme activity as compared to leaves (Fig. 2).

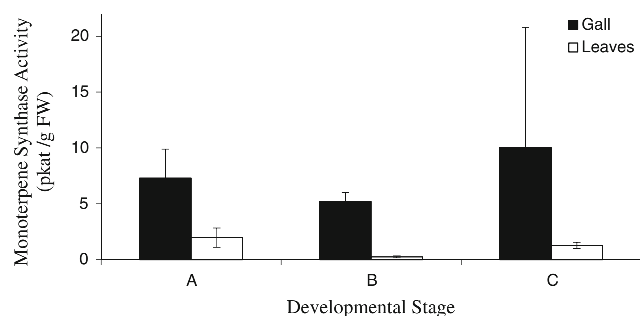


Fig. 2 Monoterpene synthase activity in soluble protein from leaves of *Pistacia palaestina* and *Baizongia pistaciae* induced galls at three developmental stages. *A*, *B* and *C* refer to the different developmental stages as indicated in Fig. 1. Amounts of producs of monoterpene synthase were calculated using radioactive geranyl diphosphate as described in Methods and Materials. Heat inactivated protein extracts were used as controls. Averages ± SE of determinations from 3 individual trees are shown. At all developmental stages, galls monoterpene synthase enzymatic activity (*black columns*) are significantly higher than that in leaves (*white columns*) ($F_{1,2} = 41.804$ $P < 0.05$)

To assess the natural polymorphism in monoterpene synthase activity levels in leaves and galls, we compared the levels of enzyme activity extracted from galls of twelve individual trees and compared to that displayed by soluble protein derived from non-colonized leaves from the same trees. Monoterpene synthase activity in galls was significantly higher than in leaves from the same tree ($T_{12.5} = -4.3$ $P < 0.001$; Fig. 3). On average, galls had four-fold higher total monoterpene synthase activity than leaves. Tree No. 9 displayed the highest difference, displaying a 12-fold increase in the monoterpene synthase activity in galls relative to leaves (Fig. 3).

Monoterpene Synthases in Galls and Leaves Produce Different Products *In Vitro* Since our initial results revealed that galls have enhanced monoterpene synthase capacities (Fig. 2, Fig. 3), we analyzed whether the products generated from geranyl diphosphate in soluble protein derived from insect-induced galls resembled those generated by similar leaf-derived enzymatic preparations. Thus, DE52-purified soluble protein extracts were incubated with geranyl diphosphate and the products generated *in vitro* analyzed by GC/MS. A marked polymorphism was apparent in the products generated in the soluble protein assays originating in different trees (Fig. 4) as expected by the high variation in monoterpene compositions of the individual trees (Rand et al. 2014). Using this GC/MS-based assay we reconfirmed our observations based on radioactive assays (Fig. 3), indicating that the monoterpene synthase activities extracted from galls yielded higher levels of monoterpenes from geranyl diphosphate than extracts derived from leaves. Moreover, there were prominent differences between the products generated by extracts from leaves and galls originating from different individual trees (Fig. 4). Based on the monoterpenes produced by enzymes in galls and leaves, two types of trees were identified. In "Type 1" trees galls have the ability to generate enhanced but similar monoterpene compositions as compared to leaves, while in "Type 2" the monoterpenes compositions generated in galls are different from those generated in extracts from leaves. For example: "Carmel 98" is a "type 1" tree whereas "Alonim" tree is a "type 2" tree (Fig. 4). In the "type 1" tree "Carmel 98" galls and leaves share common monoterpene composition patterns but exhibit only minor differences: the main monoterpene accumulated and synthesized by gall and leaves was α-pinene. The monoterpenes myrcene, limonene, and β-pinene were accumulated and synthesized prominently by galls but also by leaves albeit at lower levels than galls (Fig. 4). Enzymatic extracts derived from galls from the "type 2" tree "Alonim" catalyzed the production of α-pinene (as a major product), accompanied by lower levels of sabinene, β-pinene, myrcene, limonene, α-phellandrene, and δ-3carene. Most of these monoterpenes were absent in enzymatic assays derived from leaves of this tree. Another example of "type 2" response is displayed by "Timrat" tree, where the main compounds synthesized by soluble protein derived from galls were β-pinene, α-pinene, and β-ocimene while extracts from leaves produced lower amounts of α-pinene and β-pinene, but not β-ocimene. An additional example for "type 2" tree is "Tivon", in which the main compounds synthesized by soluble protein derived from galls were α-thujene, α-pinene, sabinene, β-pinene, myrcene, α-phellandrene, δ-3carene, and

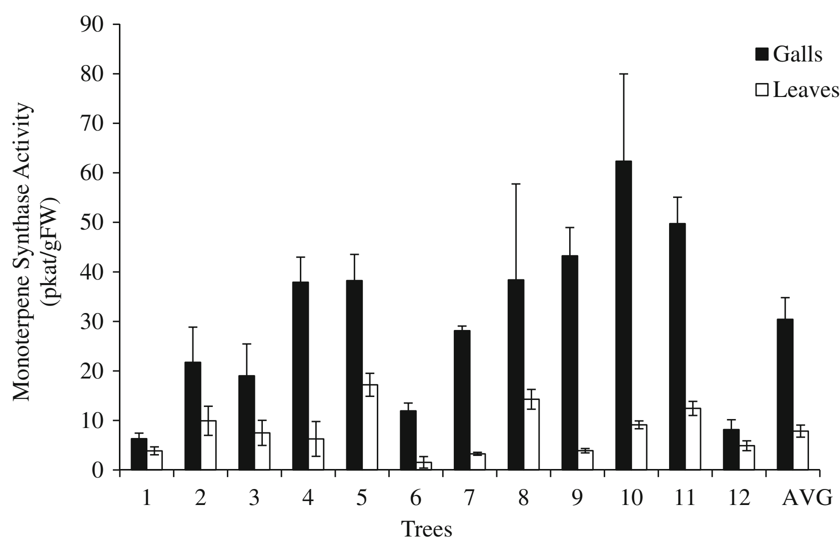
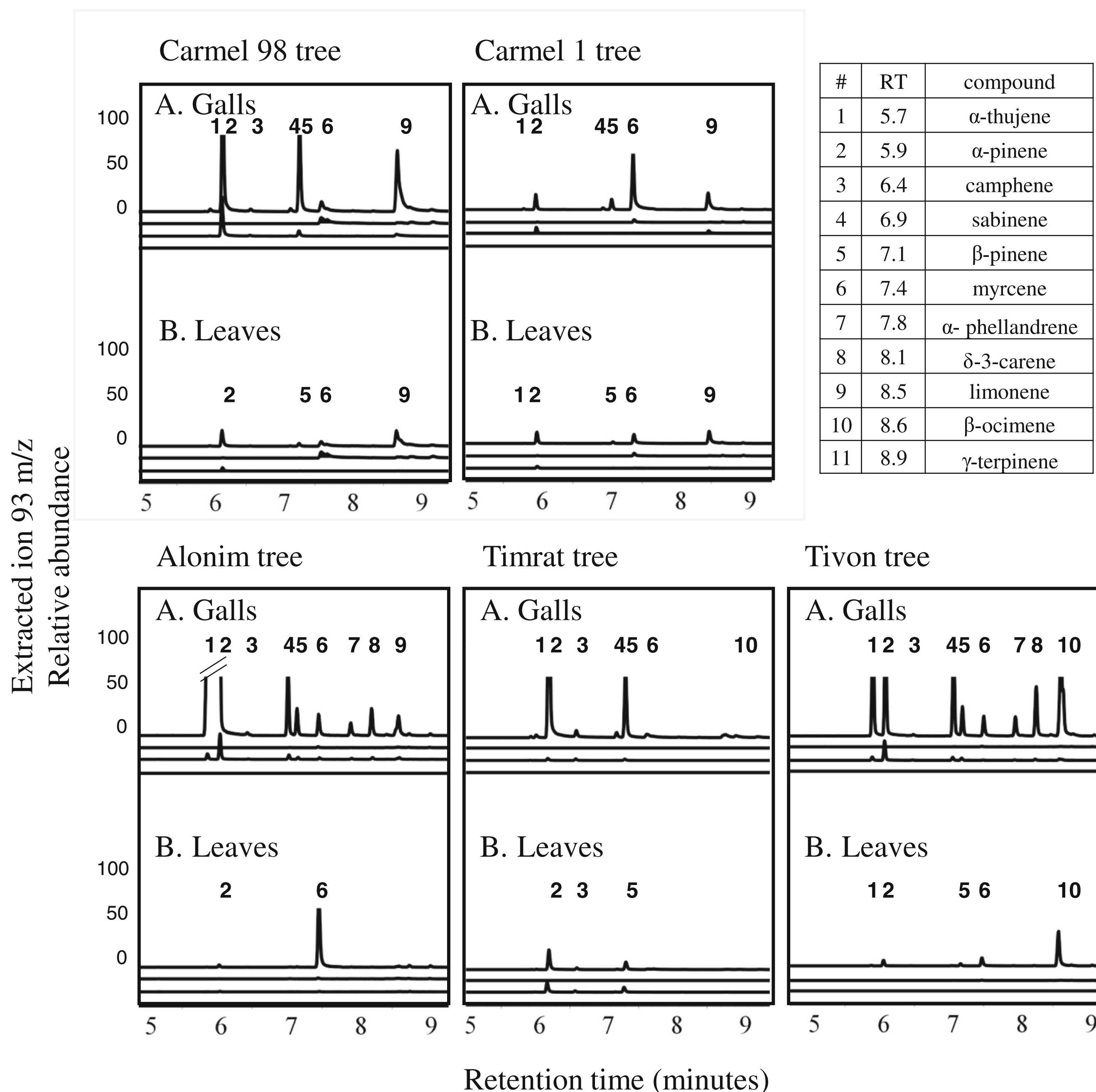


Fig. 3 Monoterpene synthase activity in soluble protein derived from *Baizongia pistaciae* derived galls and non-colonized leaves from individual *Pistacia palaestina* trees. Activity was measured by the rate of conversion of radiolabeled geranyl diphosphate into monoterpene hydrocarbons as described in Methods and Materials. Data are expressed as average of three replicates for each tree for each tissue ± S.E. Heat inactivated protein extracts were used as controls. Similar results were obtained when the enzymatic activity was expressed as specific activity on a µg protein basis (not shown). AVG indicates the averaged values for all 12 trees analyzed. Monoterpene synthase activity in galls was significantly higher than in leaves from the same tree ($T_{12.5} = -4.3$ $P < 0.001$)



#	RT	compound
1	5.7	α-thujene
2	5.9	α-pinene
3	6.4	camphene
4	6.9	sabinene
5	7.1	β-pinene
6	7.4	myrcene
7	7.8	α- phellandrene
8	8.1	δ-3-carene
9	8.5	limonene
10	8.6	β-ocimene
11	8.9	γ-terpinene

Fig. 4 Single Ion (93 *m/z*) GC/MS analyses of the products generated from geranyl diphosphate by partially purified monoterpene synthase activities isolated from galls and leaves of different *Pistacia palaestina* trees. The tree designation is shown for each panel. (**a**) Galls (**b**) Leaves. Complete assays (*upper* chromatograms in each panel), controls: protein only (mid chromatograms in each panel); controls no substrate (*bottom* chromatograms in each panel). The products generated *in vitro* from geranyl diphosphate by DE52 protein extracts were determined using GC-MS assays as described in Methods and Materials

γ-terpinene, while extracts from leaves generated much lower levels of β-ocimene, α-pinene, β-pinene, and myrcene.

Discussion

Monoterpenes are the main volatile components of *P. palaestina* oleoresin that also contains sesquiterpene hydrocarbons as well as neutral and acidic triterpenes (Caputo et al. 1979; Flamini et al. 2004; Rand et al. 2014). We have previously showed that a substantial polymorphism exists in monoterpene compositions among different *P. palaestina* trees growing in natural maquis ecosystems in Israel (Rand et al. 2014). Nevertheless, despite the polymorphism in terpene composition found among trees, we consistently observed differences in mono- and sesquiterpene levels and their ratios both as averages among trees and

specifically in individual trees between galls and non-colonized leaves. Galls consistently display enhanced monoterpene levels as compared to non-colonized leaf tissues, and the monoterpene composition in galls is generally different from those found in leaves (Rand et al. 2014). In this study we show clear evidence that aphid-induced galls produce and accumulate higher monoterpene levels than intact leaves over the course of their development (Fig. 1). Moreover, monoterpene levels in galls remain high during development on a g FW basis, while young leaves accumulated high monoterpene levels, which gradually decrease during development (Fig. 1). Taking into account that gall size and weight dramatically increase with development, our results indicate that the monoterpene level on an individual gall basis keeps increasing until the gall reaches its final size. This is in contrast to leaves that display the highest monoterpene concentrations during the earliest development stage A, after which the levels diminish on a FW basis (Fig. 1). The accumulation pattern found in leaves resembles similar accumulation patterns described for other terpenes and many other plant metabolites including defense compounds that are mainly synthesized and accumulated in young developing tissues (Bar-Peled et al. 1993; Bouwmeester et al. 1998; Lewinsohn et al. 2000; McConkey et al. 2000). This is thought to be evolutionary related to protecting tissues that are most prone to predation, infection, and abiotic stresses.

It is well known that the accumulations of lipophilic compounds (such as terpenes) normally occur in specialized anatomical structures in many plant families thus reducing the risk of toxicity to the plant itself (Fahn 1979). *Pistacia* and other species of the Anacardiaceae exhibit such structures in the form of elongated cavities, which accompany the phloem vessels and are often interconnected. The lipophilic material is stored under pressure, and thus, the system apparently has the capacity to translocate lipophilic material within the plant to seal and sterilize open wounds (Joel and Fahn 1980). Such ducts are prominent in *Pistacia* especially in galls (Álvarez et al. 2009; Kurzfeld-Zexer et al. 2015; Wool and Bar-El 1995; Wool et al. 1999). Thus, the enhanced monoterpene levels accumulated in galls could be a result of transport from adjacent or distal non-colonized tissues. Our present results provide several lines of evidence, indicating that galls induced by *B. pistaciae* possess their own biochemical capacity to produce high levels of specific monoterpenes independently to leaves, indicating that the oleoresin accumulated in insect induced galls is not merely based on transport from other tissues.

Production of specific monoterpenes in plants is mediated and often limited by the action of monoterpene synthase activities, a group of enzymes widely represented in the plant kingdom. This family of enzymes usually catalyzes the divalent-metal-mediated conversion of geranyl diphosphate into a multitude of monoterpene skeletons (Croteau et al. 2000). The levels of monoterpene synthase activity are often used as an indicator to measure the overall biosynthetic monoterpene capacity in plant tissues (Bouwmeester et al. 1998; Lewinsohn et al. 1991a, b; McConkey et al. 2000). We found that the levels of monoterpene synthase activity in soluble protein extracts derived from *P. palaestina* leaves and insect induced galls are clearly different (Fig. 2). Galls display substantially more monoterpene synthase activity than leaves at all developmental stages, but especially at developmental stages B and C when activity is still very prominent in gall tissues and lower in leaves (Fig. 2). These activity patterns largely correspond to the monoterpene accumulation profile recorded at different developmental stages (Fig. 1). This response is general as evidenced by the augmented total monoterpene synthase activity levels recorded for individual trees. Although there is a substantial polymorphism among trees in the intensity of the response to aphid colonization, all trees displayed higher total monoterpene synthase activity levels in galls than in leaves from the tree they originated (Fig. 3).

In order to biochemically rationalize the marked monoterpene polymorphism displayed by different *P. palaestina* trees (Rand et al. 2014), we determined the monoterpene products formed *in vitro* from geranyl diphosphate by protein soluble extracts that originated from individual trees. As expected, leaf chemical polymorphism is reflected by variability of products formed in the enzymatic assays (Fig. 4). Most of the protein extracts from leaf tissues produced similar monoterpene hydrocarbons from geranyl diphosphate, but the ratio of their formation in the assays was different depending on the source plant. α-Pinene was produced by all trees, while other monoterpenes such as myrcene, produced at prominent levels in tree "Alonim", was absent in protein extracts from tree "Timrat". Limonene was formed and detected in protein extracts from trees "Carmel 98" and "Carmel 1", but was apparently absent in extracts of the other trees. In contrast, β-ocimene production was prominent only in extracts from the tree "Tivon" and apparently absent in the rest of the trees (Fig. 4). Although monoterpene synthase activity has not been previously demonstrated in *Pistacia* spp., our results indicate that in this plant monoterpene biosynthesis occurs in a similar way as in other plants, as expected from the universality of the plant terpene pathway (Bohlmann et al. 1998; Croteau et al. 2000). It is likely that leaf tissues of individual *P. palaestina* trees possess different monoterpene synthase enzymes that are constitutively active or induced by leaf development, as well as other biotic or abiotic mechanisms that jointly mediate the monoterpene polymorphism observed in leaves of *P. palaestina*.

To ascertain if soluble protein extracts originating in galls have the ability to produce novel compounds as compared to extracts from leaves we compared the monoterpene profile produced *in vitro* from geranyl diphosphate using the GC/MS based assy. In all cases, enhanced monoterpene biosynthetic capacity in gall extracts was noted (Fig. 4) corroborating the radioactive assay findings (Fig. 3). Moreover, dramatic differences regarding the products formed *in vitro* in gall-derived

extracts were noted in some of the trees (Fig. 4). These differences partially provide a biochemical rationale for the differences observed in monoterpene compositions in leaves as compared to galls that originate from the same trees (Rand et al. 2014). We noted two types of trees according to their response to aphid colonization and galling based on GC/MS monoterpene synthase analysis (Fig. 4): "Type 1" response included trees with galls that produced enhanced but similar monoterpene compositions as compared to leaves, while in "Type 2" response the monoterpene compositions generated in protein extracts from galls are markedly different than those generated in extracts from leaves of the same trees. Similarly to the situation observed in leaves, in galls monoterpene formation from geranyl diphosphate is mediated by monoterpene synthase enzymes. Since these activities often produce multiple products from the geranyl diphosphate substrate, the biochemical mechanism that governs the production of a certain monoterpene are presently unknown. The difference in monoterpenes formed in assays originating both from gall and non-colonized leaf tissues of different *P. palaestina* trees (Rand et al. 2014) could be a result of the induction of novel activities at the transcriptional level or activation of existing proteins present in the plant tissues.

Variation in monoterpene composition between galls and leaves from individual trees as well as among trees was described in our previous report (Rand et al. 2014), but the biochemical rationale for this phenomenon was previously not explained. Differential terpene accumulation is not only dependent of their rate of synthesis, but may reflect possible different evaporation, emission or catabolic rates of individual monoterpenes. The variability observed in the products generated *in vitro* in assays from galls and leaves provide a good biochemical explanation for the observed different monoterpene compositions. It is clear that individual trees possess different biosynthetic capacities regarding monoterpene formation, this evidence clearly supports the notion of an independent monoterpene biosynthetic potential in aphid induced galls. Moreover, it seems that upon gall formation, distinct sets of monoterpene synthases might be induced in response to aphid challenge and gall formation and this set of induced activities is different in individual trees. It is possible that Type 1 response might be caused by augmentation of the enzymatic activity already present in leaves while Type 2 response could be due to induction or activation of a novel set of monoterpene synthase enzymes.

The number of enzymes and genes determining monoterpene composition in *P. palaestina* and the mechanism of their induction by aphids are currently unknown. Monoterpene synthases are encoded by members of the *Tps* gene family that is widely distributed in the plant kingdom (Chen et al. 2011). Polymorphism in monoterpene synthase genes influencing phenotypic monoterpene compositions has been demonstrated in many plant species (Bohlmann et al. 2000; Hall et al. 2011). It is likely that individual *P. palaestina* individuals possess and express different genes encoding for different monoterpene synthases that mediate the response. Thus, *Tps* genes present in the different genotypes, their gene expression, protein abundance or variation in catalytic efficiency towards different products (Hall et al. 2011) might dictate the final monoterpene profile produced by individual trees both in leaves and gall tissues. These phenomena, although documented in other plant species still need experimental confirmation for the relevance to *P. palaestina* and the gall forming habit. With the advent of transcriptomic data for this species, the elucidation of molecular mechanisms that underlie monoterpene formation and polymorphism as well as the response to aphids and the galling habit will be facilitated.

Terpenes have a variety of distinct ecological roles, and due to their widespread occurrence in different tissues and members of the plant kingdom, they have been referred as carrying protective or communicative roles. However, their exact ecological role is for their most part very difficult to experimentally determine (Langenheim 1994). It has been shown that the chemical components of *Pistacia* leaves and especially galls have significant impact on pathogens and predators (Gerchman and Inbar 2011; Martinez 2010; Rostás et al. 2013; Ulukanli et al. 2014). It is evident that the high levels of monoterpenes detected in this study is only part of the chemical arsenal that protect the aphids in the galls that include PR protein, tannins (Inbar et al. 1995; Rostás et al. 2013) and probably higher terpenes (Caputo et al. 1979). In fact, several studies have indicated pronounced phytochemical differences between galls and unaffected leaves of their host plants, which are probably associated with better defense and improved nutrition for the inducing insects (Allison and Schultz 2005; Nyman and Julkunen-Tiitto 2000).

A few lines of evidence shown here together with our previous studies (Rand et al. 2014) strongly imply that galls induced by the aphid *B. pistaciae* in *P. palaestina* have independent biosynthetic capacities to produce monoterpenes (see also Tooker et al. 2008). This conclusion is based on the following results; (*i*) The chemical composition of the oleoresin derived from leaves is different to oleoresin from galls (Rand et al. 2014). (*ii*). Total monoterpene synthase activity is prominent in galls and more than tenfold higher in galls as compared to leaves (Figs. 2, 3). (*iii*) Galls have a life-long ability to produce monoterpenes, in contrast to leaves, where monoterpene biosynthesis is mainly restricted to young tissues (Fig. 2). (*iv*) The monoterpenes produced *in vitro* by soluble protein extracts are different in galls and leaves (Fig. 4). This independent biosynthetic machinery is an example of the ability of the aphids (via the gall forming habit) to manipulate the enzymatic system of their host plant and recruit it for their own benefit.

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