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Metabolic profiling of *Persea americana* cv. Hass branch volatiles reveals seasonal chemical changes associated to the avocado branch borer, *Copturus aguacatae*

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ABSTRACT

Plants produce a plethora of variable metabolites that could be involved in constitutive and induced defense against herbivorous insects. Recently, metabolomics have showed to be a complementary approach in chemical ecology to screen dozens of plant metabolites, and identify those related with plant resistance or susceptibility to herbivorous insects. We compared GC–MS metabolic profiles of cambial tissue from *Persea americana* cv. Hass trees infested and non-infested by the avocado branch borer (ABB), *Copturus aguacatae*, and between undamaged and ABB damaged branches from the same tree. Using several multivariate techniques we discriminated between several overlaid dimensions of branches phytochemical variation (intraclonal, seasonal and induced response). Specifically, we found a set of sixteen highly correlated terpenoids, showing increasing proportions in borer trees as response to ABB larvae. In addition, low proportions of isoobtusilactone A and α-humulene were found in non-bored trees. The implications of dimensions of avocado phytochemical variation, and diversity as defensive traits in the interaction with *Copturus aguacatae* are discussed.

1. Introduction

Plants produce complex mixtures of plant secondary metabolites (PSM), which exhibit a striking variation. This variation is manifested at different scales in space and time, within and among individuals and populations (García-Rodríguez et al., 2012; Moore et al., 2014). Several PSM in those mixtures could be involved in plant-insect interactions as constitutive or induced defenses (Mithöfer and Boland, 2012). Furthermore, phytochemical variation can differentially determine host plant acceptance for herbivores (Schultz, 1983; Zangerl and Berenbaum, 1993). In fact, phytochemical spatiotemporal variation and diversity might be considered defensive traits for themselves (Iason et al., 2011; Nyman, 2010).

Determining the ecological role of mixtures or specific phytochemicals in plant defense is challenging due to their diversity and variation (Iason et al., 2011). Metabolomics is an unbiased technique in which metabolic profiling comparisons allow to preselect candidate compounds involved in an ecological interaction (Prince and Pohnert, 2010), therefore, reducing the number of bioassays to be done in comparison with the traditional bioassay-guided fractionation. With this metabolomic approach many plant constitutive or induced compounds related with herbivore resistance or susceptibility have been identified (Eloh et al., 2016; Jansen et al., 2009; Marti et al., 2013). However, little is known about defenses of angiosperm trees to wood-boring insects, and metabolic profiling studies about these interactions are scarce (Villari et al., 2016). The aspects of chemical ecology of wood-borers are relevant because some of them are invasive pests which cause high economic losses in comparison with sap or foliage feeders (Aukema et al., 2011).

The interaction between *Persea americana* cv. Hass with the branch borer *Copturus aguacatae* is a pertinent model to study phytochemical variation and its role in plant defense. Hass avocado is a hybrid between *P. americana* var. *drymifolia* and *P. americana* var. *guatemalensis*, which is propagated mainly by cloning (Rincón-Hernández and Espinosa-García, 2008). Thus, low phytochemical variation among clones would be expected. However, remarkable variation in foliar terpenoids have

Abbreviations: GC–MS, gas chromatography-mass spectrometry; PLS-DA, partial least squares discriminant analysis; ABB, avocado branch borer; B, bored; NB, non-bored; NwD, new damage; OlD, old damage; UnD, undamaged; AUROC, area under de receiver operating characteristic; VIP, variable importance in the projection

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been reported in orchards from the "Avocado Belt" in Michoacán, México, where 3 to 13 chemotypes can be found in a single orchard; some of those chemotypes were associated differentially with several avocado pests (Espinosa-García et al., 2001; García-Rodríguez et al., 2016). Many phytochemicals from *Persea americana* have shown anti-herbivore activity such as terpenoids, phenylpropanoids, acetogenins and avocadofurans (Torres-Gurrola et al., 2016).

The avocado branch borer (ABB), *Copturus aguacatae* Kissinger (Colecoptera: Curculionidae), is a Mexican endemic specialist insect on avocado trees, particularly in all varieties of *Persea americana*. It is one of the five worst quarantine pests of Hass avocado in México because it causes fruiting branches breakage and its presence prevents fruit exportation (SENASICA, 2010). Adult insects feed on shoots and leaves while larvae bore galleries in branches. In areas above 1800 masl, there is only one generation per year (Coria et al., 2007) where adults are present during the rainy season (from beginning of June to end of September) and larvae are present during the dry season (October to May). The aspects related to chemical ecology and host choice of the ABB had not been studied, and there are no identified pheromones for this weevil. Research in this field is important to develop management strategies of this potentially invasive pest (Luna et al., 2017). In orchards from the "Avocado Belt" in Michoacán, México, we have observed differential infestation of ABB, that is, while some trees are severely infested, other neighboring trees remain non-infested. Indeed, avocado rare chemotypes showed higher ABB incidence than common chemotypes (Espinosa-García et al., 2001). These observations suggest a relationship between avocado chemistry and some kind of resistance (antibiosis or antixenosis) to ABB. Thus, we hypothesized that variation within the Hass cultivar in leaf and branch secondary compound profiles explain differential infestation by *C. aguacatae*. We sampled 130 trees of ABB infested trees and their neighboring non-infested trees in seven orchards, and we analyzed leaf and branch volatile lipophilic compounds by GC–MS to determine whether infested and non-infested individuals were chemically different.

In order to distinguish between constitutive and induced compounds, in a second field study, we compared chemical profiles of borer infested and non-infested branches from the same tree. Additionally, we contrasted the chemical profiles from infested branches containing ABB larvae and branches with empty galleries as a proxy to induced response or mechanical damage, respectively. We compared the metabolic profiles by multivariate analysis to determine which compounds (constitutive or induced), alone or in mixtures, were related to ABB presence or absence. Non-infested trees were visited two years later to determine if they remained uninfested.

2. Materials and methods

2.1. First field study and sampling

In a first study, seven orchards of Hass avocado in Villa-Madero and Tingambato municipalities were visited within the Michoacán "Avocado Belt" in México (Table 1). These municipalities are under an official campaign to eradicate avocado branch borer; phytosanitary brigades actively saw-prune ABB infested branches within affected orchards, but sometimes neighboring orchards that may be infested are not treated unless the owners authorize the treatment. Twenty trees per orchard were sampled (except in two orchards in which non-infested trees were scarce), ten infested with *C. aguacatae* larvae and ten neighboring trees with no evidence of infestation. Borer-infested branches exude perseitol that dries into a powdery white dust outside of the bore left by the larvae (SENASICA, 2010). Also, saw-pruned trees were recognized as susceptible to the branch borer. Two or three branches of 1–3 cm width and 40 cm long were cut, stored in plastic bags, and maintained at 5 °C until analysis. Samples were taken from tertiary branches (1–3 cm) in the middle tree canopy (2–4 m) where the major weevil infestation occurs (Coria et al., 2007). Additionally, mature leaves from sampled branches were taken and stored in plastic bags for tree chemotype determination. The samples were taken regardless branch orientation as the incidence of infested branches was the same towards any cardinal point (Coria et al., 2007). Sampled trees were labeled and two years later they were visited again to determine if some trees are immune (remain non-infested), have some kind of resistance (slow ABB colonization or suppressed infestation) or susceptible (finally infested and severely infested). Another set of infested branches was collected and maintained in plastic bags until adult insect emergence. Adults were identified as *Copturus aguacatae* Kissinger (Muñiz and Barrera, 1958). Two orchards (2 and 4) were resampled (ten trees for each one), one for each municipality to get a complete view of phytochemical variation.

Table 1
Sampled Hass avocado orchards.

No	Orchard name	Municipality	Coordinates	Altitude (masl)	Sampling month
1	Derrumbadero	Villa Madero	N 19°23'38.3"; W 101°18'00.1"	2284	June
2	Terencio	Villa Madero	N 19°21'59.9"; W 101°16'02.2"	2252	June
3	El chirimo	Villa Madero	N 19°22'26.9"; W 101°15'53.1"	2055	September
4	Ojo de agua	Tingambato	N 19°29'44.8"; W 101°52'18.1"	1898	December
5	La fábrica	Tingambato	N 19°30'19.9"; W 101°50'55.0"	2012	December
6	El serrato	Tingambato	N 19°30'13.9"; W 101°50'44.5"	2023	March
7	El tanque	Tingambato	N 19°29'22.6"; W 101°50'27.4"	2022	March

2.2. Second field study

In order to determine if compounds showing differences between bored and non-bored trees in the first field study were constitutive or induced, a second field study was conducted. Three types of branches were sampled from ABB infested trees: undamaged branches (UdB), with recent ABB damage (containing active larvae and showing the characteristic white exudate, NwD) and with ABB old damage (only empty galleries, OlD). Tree branches of each type from six trees from orchard 1 were sampled, and lipophilic volatile profiles were obtained in the same way that in the first field study.

2.3. Sample processing

The cambium tissue was removed from each branch, avoiding necrotic tissue, with a knife and separated in two parts; leaves were cut on the middle vein. One half of each tissue (leaf or branch) was dried at 80 °C to obtain dry weight. The other half was cut in small fragments with scissors and put in a 75 mL amber flask with 40 mL of analytical grade hexanes and 1.0 mg of n-tetradecane as an internal standard. Samples were stored at 4 °C for a week. Then, cambium (~5 g FW) or leaf (~2 g FW) tissue of each tree was ground with neutral sand and hexanes. Grinding with periodical hexanes removal and fresh hexanes addition was repeated until the hexanes extract was clear. The collected extract was filtered, dried over anhydrous sodium sulfate and concentrated under a N_2 current until 1.0 mL. For each tree a leaf and a branch extract were obtained from composite samples of three leaves or three branches.

2.4. GC–MS analysis

Each extract was homogenized in a vortex for 15 s and then 1 μL was injected into an Agilent HP6890 gas chromatograph equipped with a