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Longitudinal study of Caribbean pine elucidates the role of 4-allylanisole in patterns of chemical resistance to bark beetle attack

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Research Article

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Abstract

Southern pine beetles (*Dendroctonus frontalis* Zimmermann) and symbiotic fungi are associated with mass mortality in stands of Caribbean pine (*Pinus caribaea* Morelet). This study provides a 12.7-year assessment of semiochemical mediation between southern pine beetle and Caribbean pine in relation to concentrations of 4-allylanisole (estragole, methyl chavicol) and monoterpenes measured by gas chromatography–mass spectrometry in different seasons in premontane and coastal pine stands of Belize and Guatemala. Individual trees and stands with >2.5% (relative mass %) of 4-allylanisole in the xylem oleoresin exhibited significantly less beetle-induced mortality than those with <2.5%. Changes in relative levels of 4-allylanisole and monoterpenes during this study are consistent with seasonal temperature and cumulative water deficit effects and suggest bark beetle attack of *P. caribaea* may intensify in the future.

Introduction

Recent increases in global temperatures and shifts in precipitation worldwide have disrupted the longstanding equilibrium between conifers and bark beetles. Higher annual temperatures and shorter winters extend and enhance beetle reproduction, causing additional stress to trees that beetle larvae use as a food source (Bentz & Jönsson 2015). In pine forest ecosystems, bark beetles contribute to the welfare of plant communities through nutrient cycling, canopy thinning and biodiversity generation (Glare *et al.* 2011). Conversely, beetle-induced mass mortality dampens forests' capacity for water purification, slope stabilization, carbon sequestration, wildlife conservation and soil formation (Kurz *et al.* 2008). Because of these impacts, measurements of the effect of climate change on host trees' natural defences against bark beetles are especially important. Tree growth rates are usually so slow that space-for-time proxies, such as altitude gradients, are often used in climate change forecasting (Ferrenberg *et al.* 2017). In this investigation, we attempt a direct measurement of changes in relative percentages of 4-allyallanisole (estragole, methyl chavicol) in Caribbean pine (*Pinus caribaea* Morelet) using a longitudinal study of xylem oleoresin volatiles related to bark beetle attack that spans 12.7 years.

Southern pine beetles (SPB, *Dendroctonus frontalis* Zimmermann) and possibly *D. mesoamericanus* Armendáriz-Toledano and Sullivan (Armendáriz-Toledano *et al.* 2015) have significantly affected Caribbean pine populations in Central America. SPB is predicted to kill 439 000 km^2 of pine forests by the year 2080 (Katz 2017). Caribbean pines can produce enough defensive oleoresin and resin flow to ward off beetle attack. However, especially under stressful conditions such as drought, trees often succumb to larval feeding on the phloem and to symbiotic fungi that grow from spores carried by female beetles in their mycangia.

The phenylpropanoid 4-allylanisole (1-methoxy-4-(prop-2-en-1-yl)-benzene, CAS #140-67-0, hereafter 4-AA) has received attention as a semiochemical mediator between bark beetles and pine trees (Werner 1995). It is produced by a different synthetic pathway than terpenes, and it appears to be repellent to various species of scolytid beetles including SPB (Hayes *et al.* 1994). Higher xylem oleoresin levels of 4-AA have been correlated with lower attack by *Dendroctonus ponderosae* Hopkins in *Pinus ponderosa* var. *scopulorum* Engelmann (Emerick *et al.* 2008, Hobson 1995). In addition, sufficient levels of 4-AA deter fungal growth, allowing an attacked tree to survive the fungal infections that arrive with bark beetles (Hofstetter *et al.* 2005).

In previous analyses of 4-AA in the monoterpene fraction of Caribbean pines of the Mountain Pine Ridge (MPR) Forest Reserve in Belize, Snyder & Bower (2005) found significantly more 4-AA (>2.5%) in the xylem oleoresin of low-mortality (little or no visual evidence of beetle-induced mortality) stands of *P. caribaea* var. *hondurensis* than in high-mortality

($\geq$90% mortality) stands that had sustained high levels (median of 35 pitch tubes per tree) of SPB attack. Furthermore, levels of 4-AA > 2.5% in individual trees were found to be negatively correlated with the magnitude of beetle attack, quantified as the number of pitch tubes per tree.

There are at least three varieties of *P. caribaea*: *P. c.* var. *hondurensis*, *P. c.* var. *bahamensis* and *P. c.* var. *caribaea*. Coppen *et al.* (1993) analysed the xylem oleoresin of these varieties grown in SPB-free plantations from seeds obtained from nine Caribbean locations. The varieties exhibited different constitutive levels of 4-AA, with the *P. c. caribaea* and *P. c. bahamensis* varieties averaging below the 2.5% threshold (1.3%, and 1.5% to 2.2%, respectively). Furthermore, *P. c. hondurensis* grown from seeds obtained from coastal sites in Belize and Nicaragua had average levels at or below 2.5% (2.5% and 1.6%, respectively). These differences, coupled with the findings of Snyder & Bower (2005) at MPR and a predicted increase of 0.32°C and a decrease of 1.2% in precipitation per decade in Belize (Richardson 2009), suggested a longitudinal study of 4-AA levels in indigenous stands of *P. c. hondurensis* should be conducted to determine if these climate variables might impact the levels of monoterpenes and 4-AA that mediate beetle attack.

Methods

In 2007, three coastal stands of *P. caribaea* var. *hondurensis* without visible signs of SPB attack were sampled. Given the patterns found in the MPR forests studied in 2004 (Snyder & Bower 2005), trees in the Forest Reserves of Mango Creek I (MC1), Mango Creek IV (MC4) and Swasey-Bladen (SB), were predicted to have average levels of 4-AA > 2.5% in the oleoresin's monoterpene fraction, potentially protecting them from SPB attack. In addition, two stands of *P. c. hondurensis* in premontane forests of Guatemala with two levels of beetle attack were sampled in 2008.

Our results from 2004 suggested that stands with >2.5% 4-AA should resist SPB attack. In this longitudinal study, trees from two of the three 2007 coastal sites (SB and MC4) were resampled in 2016 for their monoterpenes and 4-AA and evaluated for their mortality levels. (MC1 had human encroachment that prevented resampling.) The samplings in March 2004 (25.3°C; 4.7 cm rain/month), March 2007 (23.9°C; 4.3 cm rain/month), August 2008 (20.5°C; 35 cm rain/month) and November 2016 (25.0°C; 22 cm rain/month) included stands that were paired with high and low levels of attack, wet and dry seasons, and coastal (~40 m) and premontane (~500 m) elevations.

Samples were collected for chemical analyses by drilling 1.2 cm holes into trees with diameters at breast height (dbh; 1.2 m) between 14 and 43 cm. Holes were drilled at a slight upward angle. Sample vials were immediately inserted into the predrilled holes and left to collect xylem oleoresin for 24 hours. The vials were removed, capped, and subsequently refrigerated until analysed. Samples were analysed for their 4-AA and monoterpene composition using GC-MS (Agilent 7890A-5975C) with 30 m HP-624 and (cross-calibrated) HP-5MS columns. Samples were diluted with heptane and analyses were conducted on the HP-5MS using total-ion chromatography and the 68, 93 and 148 ions with the temperature programme: 10 min at 50°C, followed by a 15 min temperature ramp to 250°C. This procedure resolved and quantified 4-AA and 10 monoterpenes. The data were converted to relative mass percentages using authentic standards, and identities of the components were verified via the mass spectra and retention times. Data analyses were performed using Minitab ver. 18.1 (Minitab Inc., State College, PA, 16801, USA).

Results and Discussion

Table 1 summarizes the relative percentages of the analysed compounds in the oleoresin. Higher levels of chemical diversity within the host and surrounding plants have been shown to reduce insect damage (Richards *et al.* 2015). Monoterpene diversity was quantified with the diversity index, $H'_m = -\Sigma p_i \ln(p_i)$, where p_i = the proportion of each of the 10 monoterpenes. In this study, H'_m was inversely correlated with α- and β-pinene (Spearman $r_s^2 = 0.77$ and 0.20 respectively, $P < 0.00001$, n = 305 trees).

Recently, Ferrenberg *et al.* (2017) found H'_m increased with *P. ponderosa* age. We found a similar increase related to dbh for H'_m and % 4-AA in *P. caribaea* ($r_s^2 = 0.15$, $P = 0.002$; and $r_s^2 = 0.22$, $P = 0.0001$, n = 62, respectively) at Mountain Pine Ridge during the dry season. Adding wet season data from comparable elevations in Guatemala suggests % 4-AA reaches a maximum near dbh = 30 cm and then marginally declines. The combined data (n = 102 trees) also suggest H'_m is more independent of size during wet seasons.

Using only the linked, paired data sets and after removing five outliers (three MPR and two SB pines with >80% α-pinene), the H'_m data met the assumptions of normality and equality of variances for a three-factor, two-level GLM ANOVA of high versus low attack status, high versus low altitude and wet versus dry seasons. (Because of their collinearity, temperature, precipitation and time could not be used as separate predictors.) H'_m was found to be significantly higher during warm and dry periods (F = 92.2, $P < 0.00001$, n = 239). Attack status and altitude were not significantly related to H'_m. While Ferrenberg *et al.* (2017) found H'_m decreased with elevation in some pine species, their study involved a wider range of higher elevations (1800 – 3300 m).

An arcsine, square root transformation successfully normalized percentages of 4-AA without rejecting outliers. As predicted, the three-factor, two-level GLM ANOVA of attack status, altitude and season for paired sites found stands of *P. caribaea* that avoided mass-mortality from beetle attack over the course of this study (Figure 1) had higher relative percentages of 4-AA (4.04% *vs* 2.13%; F = 28.60, $P < 0.00001$, n = 245). Furthermore, altitude, season and interactions were all insignificant for 4-AA ($P \geq 0.3$). Even higher levels of significance were found including unpaired trees from the MC1 stand with Mood's median tests for 4-AA *vs* attack status ($\chi^2 = 24.91$, $P < 0.00001$, n = 305), and for season *vs* H'_m ($\chi^2 = 55.05$, $P < 0.00001$, n = 305). While H'_m and 4-AA could be transformed to meet the assumptions of a GLM ANOVA, individual monoterpenes often did not meet these assumptions. Mood's median test makes no assumptions about normality or equality of variances, so it was used to examine individual monoterpenes. Except for a positive association with β-pinene ($\chi^2 = 11.26$, $P = 0.0008$, n = 305), none of the monoterpenes was significant with respect to beetle attack after Bonferroni correction.

Altitude, attack status, rainfall and temperature (which was inversely correlated with rainfall) are not fully orthogonal factors in the unbalanced experimental design, so the significance of factors extracted using a GLM ANOVA must be treated cautiously. The reported temperature increase and rainfall decrease during this 12.7 year study are +0.22°C and −2.6% (World Bank Group 2019). Based on work such as Ferrenberg *et al.* (2017), we expected our study to find inverse correlations between 4-AA

Table 1. Summary of the mean relative percentages of xylem oleoresin monoterpenes and 4-AA and their standard errors (SE), sample sizes, and the measured chemodiversity of *P. caribaea*

Locations:	Premontane sites		Coastal sites	
Treatments:	High Attack	Low Attack	2007	2016
No. of stands:	4	4	3	2
No. of trees:	47	41	177	40
Compounds	Mean ± 1 SE	Mean ± 1 SE	Mean ± 1 SE	Mean ± 1 SE
thujene	0.34 ± 0.05	0.37 ± 0.06	0.50 ± 0.04	0.13 ± 0.02
α-pinene	62.26 ± 9.97	60.89 ± 10.44	58.54 ± 4.40	63.81 ± 10.09
camphene	1.19 ± 0.19	1.11 ± 0.19	1.25 ± 0.09	0.99 ± 0.16
sabinene	0.23 ± 0.04	0.18 ± 0.03	0.22 ± 0.02	0.22 ± 0.03
β-pinene	5.81 ± 0.93	4.47 ± 0.77	4.22 ± 0.32	3.69 ± 0.58
myrcene	2.62 ± 0.42	2.54 ± 0.44	1.95 ± 0.15	1.56 ± 0.25
α-phellandrene	0.83 ± 0.13	0.84 ± 9.14	0.85 ± 0.06	0.69 ± 0.11
limonene	1.45 ± 0.23	1.47 ± 0.25	3.69 ± 0.28	2.17 ± 0.34
β-phellandrene	23.46 ± 3.76	24.51 ± 4.20	24.24 ± 1.82	22.83 ± 3.61
terpinolene	0.14 ± 0.02	0.14 ± 0.02	0.31 ± 0.02	0.14 ± 0.02
4-allylanisole	2.13 ± 0.34	4.04 ± 0.69	4.00 ± 0.30	3.47 ± 0.55
Shannon's *H'*	1.17 ± 0.04	1.14 ± 0.03	1.21 ± 0.02	1.09 ± 0.03
Σ of majors (>5%)	85.72 ± 10.65	85.72 ± 10.65	85.72 ± 10.65	85.72 ± 10.65
Σ of lows (0.5–0.5%)	14.03 ± 1.13	14.47 ± 1.17	15.96 ± 0.55	12.58 ± 0.93
Σ of minors (0.1–0.5%)	0.71 ± 0.07	0.69 ± 0.07	1.03 ± 0.05	0.49 ± 0.05
Trace (<0.1%)	(Detected, not quantified: 3-carene, p-cymene, γ-terpinene and ocimene)			

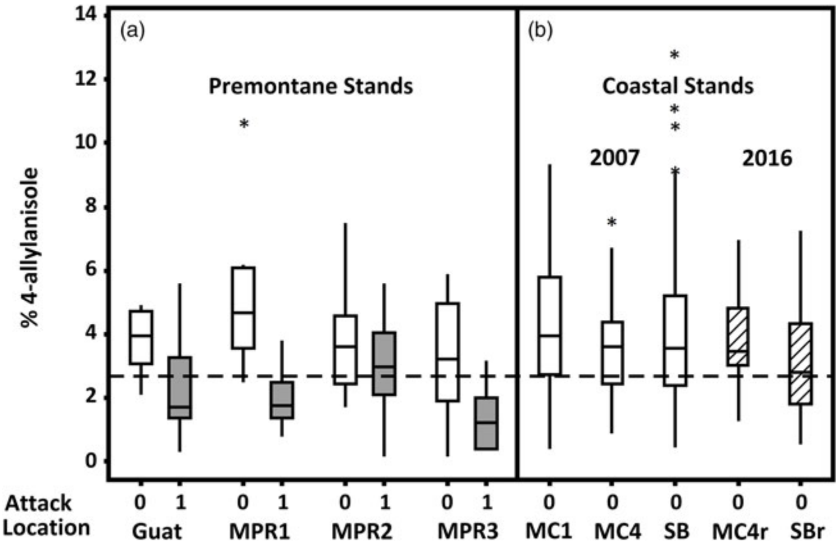

Figure 1. Box plot displaying the variation of 4-AA (a) between low-attack (0) and high-attack (1) stands in premontane sites sampled in 2004 (MPR) and 2008 (Guatemala), and (b) between the coastal 2007 and 2016 repeated (r) samplings. Boxes = inner quartiles; whiskers = max and min within 1.5 × of box height; lines within boxes = medians, asterisks are outliers. Dashed line = the 2.5% threshold. Shaded stands had significantly higher levels of mortality ($\chi^2 = 20.14$, $P < 0.00001$, n = 88). The 2016 stands (hatched) showed a borderline significant decline in 4-AA ($\chi^2 = 4.28$, $P = 0.039$, n = 217) compared with 2007 that may be due to factors such as seasonal variation and/or increasing stress from climate extremes.

and altitude, attack status and time, but a direct correlation with rainfall. Based on all 305 trees, only attack status was significantly associated with 4-AA.

Because many compounds are summarized by H'_m, only some of them defensive, the correlations with H'_m are harder to predict. Still, we expected to find H'_m inversely related to altitude, attack status and rainfall, and directly related to temperature. Only rainfall (and/or temperature) was significantly associated with H'_m.

Changes in H'_m found in this study are within the normal range of plant plasticity for the compounds analysed. Lower H'_m values in this study correspond to higher levels of monoterpenes such as α-pinene, β-pinene and/or myrcene that act synergistically with beetle pheromones as attractants (Sullivan 2016). Beetle antenna analyses have shown that SPB responds to α-pinene, β-pinene, myrcene and 4-AA (Sullivan 2016). High levels of 4-AA inhibit the growth of symbiotic fungi (Marei *et al.* 2012), so it may act as a deterrent to pioneering beetles. 4-AA may also be directly toxic to the beetles (Werner 1995). Thus, increases over time of the relative percentages of α-pinene, β-pinene and myrcene, coupled with decreases in 4-AA due to dilution by monoterpenes when higher temperatures and/or cumulative water deficits increase resin flow (Blanche *et al.* 1992), are likely to intensify future beetle attacks. Such dilution effects may explain what appears to be a modest decline in 4-AA over time shown in Figure 1.

Outside of the seasonal monoterpene dilution effect, the essentially constant level of 4-AA found in this study is consistent with the idea that 4-AA levels in *P. caribaea* are constitutive. Stressors such as photochemical smog have been correlated with lower levels of 4-AA released from ponderosa pine needles and higher levels of beetle attack. However, xylem oleoresin levels were constant (Cobb *et al.* 1972). The different levels of 4-AA found by Coppen *et al.* (1993) in the *P. caribaea* varieties free from SPB attack also suggest 4-AA is constitutive. The lowest mean levels (<2.0%) are found in Caribbean pine varieties from the Bahamas and Cuba while the highest levels (>2.5%) are found in Belize and Guatemala where populations of *P. caribaea* have been subject to selective pressure from SPB attack. Similar selection has been suggested for mountain pine beetle (Six *et al.* 2018).

This study expands earlier findings (Snyder & Bower 2005), suggesting 4-AA is a constitutive component that mediates SPB attack in *P. caribaea*. It also suggests that trees with insufficient levels of this semiochemical, such as from the use of inappropriately sourced seed stock and from stressors such as large shifts in temperature and rainfall, are likely to make stands of *P. caribaea* more susceptible to mass mortality from future bark beetle attacks.

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