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Differential Response in Foliar Chemistry of Three Ash Species to Emerald Ash Borer Adult Feeding

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Abstract The emerald ash borer (EAB; *Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae), is an exotic wood-boring beetle that has been threatening North American ash (*Fraxinus* spp.) resources since its discovery in Michigan and Ontario in 2002. In this study, we investigated the phytochemical responses of the three most common North American ash species (black, green, and white ash) in northeastern USA to EAB adult feeding. Black ash was the least responsive to EAB adult feeding in terms of the induction of volatile compounds, and levels of only two (indole and benzyl cyanide) of the 11 compounds studied increased. In green ash, levels of two [(*E*)-*β*-ocimene and indole] of the 11 volatile compounds studied were elevated, while the levels of two green leaf volatiles [hexanal and (*E*)-2-hexenal] decreased. White ash showed the greatest response with an increase in levels of seven of the 11 compounds studied. Qualitative differences among ash species were detected. Among the phenolic compounds detected, ligustroside was the only one detected in all three species. Oleuropein aglycone and 2 unidentified compounds were found only in black ash; coumaroylquinic acid and feruloylquinic acid were detected only in green ash; and verbascoside hexoside was detected only in white ash. EAB adult feeding did not elicit or decrease concentrations of any selected individual phenolic compounds. However, although levels of total phenolics from black and green ash foliage were not affected by EAB adult feeding, they decreased significantly in white ash. EAB adult feeding elevated chymotrypsin inhibitors in black ash. The possible ecological implications of these findings are discussed.

Key Words *Agrilus planipennis* · *Fraxinus* · Plant defense · Phenolics · Volatile organic compounds · VOCs · Coleoptera · Buprestidae

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Introduction

Many volatile and non-volatile phytochemicals have various ecological and physiological functions in interactions with biotic and abiotic factors. Some adversely affect colonization or development by herbivores (Berenbaum 1995; Duffey and Stout 1996), while some function as herbivore attractants or attractants for natural enemies of herbivores (Dicke et al. 1990; Turlings et al. 1991). In response to herbivore damage, many of these compounds can accumulate in plant tissues (Karban and Baldwin 1997; Chen et al. 2008, 2009) with significant effects on herbivore interactions with conspecifics and heterospecifics (Agrawal and Karban 2000). For instance, increased density of pigment glands (containing terpenoids such as gossypol) of cotton leaves induced by strawberry mites, *Tetranychus turkestani* Ugarov & Nikolskii (Prostigmata: Tetranychidae), significantly reduced beet armyworm growth [*Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)] (Agrawal and Karban 2000). Beet armyworm larvae also can distinguish between previously damaged and intact

(control) cotton leaves and will consume more control leaves than previously damaged leaves in dual-choice tests (McAuslane et al. 1997).

The exotic emerald ash borer (EAB; *Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae) has been threatening North American ash (*Fraxinus* spp.) trees since its discovery in Michigan and Ontario in 2002 (MSU 2010). Although North American ash species, such as black ash (*F. nigra* Marshall), green ash (*F. pennsylvanica* Marshall), and white ash (*F. americana* L.) are all susceptible to EAB compared to the Asian ash (e.g., Manchurian ash, *F. mandshurica* Ruprecht) (Rebek et al. 2008), evidence points to inter-specific variation in constitutive foliar chemistry among these North American species (Pureswaran and Poland 2009). Differences in feeding and infestation preference in laboratory studies appear to be associated with such phytochemical variability (Pureswaran and Poland 2009, Chen and Poland 2010). Intact black, green, and white ash release many volatiles that trigger electro-antennal responses of EAB adults (Rodriguez-Saona et al. 2006; de Groot et al. 2008) and possess a wide variety of phenolics (Eyles et al. 2007) and protease inhibitors (Chen and Poland 2009b) that may confer resistance against EAB. Phenolics generally denature plant proteins and thus reduce nutritive values of plants (Dudt and Shure 1994; Ossipov et al. 2001). Phenolics also can inhibit the absorption of key amino acids. Many protease inhibitors are involved in herbivore defense (Ryan 1990; Zavala et al. 2004). Protease inhibitors impair herbivore growth by eventual depletion of essential amino acids that result from an herbivore's compensatory production of digestive proteases (Broadway and Duffey 1986).

Many phytochemicals can be induced by herbivore damage. However, little is known about the physiological response of North American ash species to EAB adult feeding. Differential responses of ash species to EAB adult feeding may change the dynamics of ash foliar phytochemicals, which can further affect the behavior of EAB, other herbivores of ash trees, and potential natural enemies of EAB larvae. Elucidating inter-specific variation among these compounds also might contribute to understanding differences in feeding and infestation preference among North American species. Our objectives in this study were to determine: (1) the quantity and composition of volatile organic compounds (VOCs), polyphenols, and trypsin and trypsin inhibitors of the three ash species in response to EAB adult feeding; (2) if the magnitude of induction differs among the three species.

Methods and Materials

Ash Seedlings Green, white, and black ash seedlings (2-yr-old and ca. 30–50 cm tall) were purchased from Lawyer Nursery Inc. (West Plains, MT, USA) in February, 2009. Trees were stored in a dark room at 4°C until potting. Seedlings were potted in TPOT2 tree pots (width: 15 cm; height: 41 cm; volume: 6.23 L; Stuewe & Sons, Inc., Corvallis, OR, USA) using Fafard #52 soil (BFG Supply Co., Burton, OH, USA) as planting medium. They were grown during April-May, 2009 (ca. L14: D10), in a greenhouse set at approximately 25±2°C. The greenhouse is located at the Tree Research Center, Michigan State University, East Lansing, MI, USA. Seedlings were fertilized once every 3-4 day with approximately 2 L nutrient solution containing 200 ppm N, 60 ppm P, 150 ppm K, 80 ppm Ca, 40 ppm Mg, 60 ppm S, 0.15 ppm Cu, 4 ppm Fe, 0.8 ppm Mn, and 0.32 ppm Zn.

EAB Adult Females EAB adult females were collected from 90-cm long logs cut from naturally infested ash trees felled at the end of 2008 and early 2009 in Ingham County, MI, USA. Logs were held in cold storage at 4°C until needed, then removed to allow beetles to emerge. Beetles were separated by sex and kept in 295 ml plastic beverage containers with an evergreen ash, *F. uhdei* (Wenzig) Linglesh, leaf in a vial of water for feeding. Containers with beetles were stored in growth chambers (25°C) with a photoperiod of 14:10 hL:D, and >70% relative humidity. Leaves were replaced twice a week, until needed for experiments.

Headspace Leaf Volatile Collection and Analysis Constitutive headspace volatile profiles of the three species used were investigated in Pureswaran and Poland (2009). Headspace leaf volatiles of EAB damaged leaves (a whole branch from "induced treatment", see below) and those without EAB damage (a whole branch from "control treatment") of the same ash species attached to live seedlings were collected and compared in this study.

EAB treated leaves were prepared by caging a branch bearing 5–8 mature compound leaves within a nylon sleeve with both ends tied. Mature leaves were chosen because EAB adults are known to prefer mature over young foliage (Chen and Poland 2009b). To simulate common levels of foliage damage in nature by EAB, adults (personal observations), ten 8- to 10-d-old adult females were placed inside the sleeve and allowed to feed for 48 h. Adult females then were removed and frass gently brushed from the leaves. Approximately 5–10% of the foliage was visually estimated to be consumed within the 48 h ("induced treatment"). Control leaves also were enclosed by a sleeve but without beetles ("control treatment").

The volatile compounds of the whole branch, either from "induced treatment" or "control treatment", subsequently

were collected with a push-pull system described in Rodriguez-Saona et al. (2006). Briefly, the branch was enclosed in a 4 L glass cylinder (height: 30.5 cm; diam: 15.2 cm; Analytical Research Systems, Inc., Gainesville, FL, USA). Activated charcoal-purified air generated by a pump entered the top of the glass cylinder at a rate of 2 L/min. Half of the air with a rate of 1 L/min passed through a volatile collection trap (VCT) located 5 cm above the base of the cylinder by a vacuum pump. The VCT was filled with 30 mg Alltech Super-Q absorbent material. Volatiles were collected in the laboratory under 25±1°C with about 40% RH for 24 h. The room was illuminated with four 40 W fluorescent light tubes. At the end of volatile collection, the aerated portion of the seedling was clipped, and dry weights (oven-dried at ca. 60 C) of aerated tissue were recorded. Each treatment (EAB vs. control) was replicated 8 times. Volatile collection was conducted separately by species (i.e., black, green, and white ash) due to the limitation of available volatile collection systems. Volatile collection was conducted approximately 4 week after potting of the seedlings.

Methods of volatile extraction and analysis are described in Chen and Poland (2009a). Collected volatiles were extracted from the Super-Q adsorbent by flushing with 250 μl of pentane-hexane (both HPLC grade; purity: pentane >99%; hexane: ≥ 95%) mixture (1.5:1 ratio) containing 1 ng μl^{-1} of heptyl acetate (internal standard; purity >98%) with a stream of nitrogen gas. Two μl of each sample were injected into a Thermo Scientific Trace GC gas chromatograph equipped with a DSQ-II MS mass spectrometer and a 30 m × 0.25 mm id, 0.25 μm thick TR-1MS column by using splitless injections and helium as the carrier gas. The oven temperature began at 60°C for 1 min, increased by 10°C/min to 190°C, then by 35°C/min to 300°C, and held for 5 min. The following 11 compounds were detected consistently: hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, cubenene, (*E*)-β-ocimene, indole, benzyl cyanide, and nonanal. They were quantified based on comparison of peak areas with that of the internal standard and calibration curves generated with synthetic standards. The purity of these synthetic standards was 98% (hexanal), 98% [(*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene], 70% [(*E*)-β-ocimene and indole], 99% (benzyl cyanide), and 95% (nonanal). The amount of each compound of interest was calculated in ng g^{-1} dry weight of tissue. All reagents and solvents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of Non-volatile Defensive Compound We examined the following non-volatile foliar defense responses to EAB adult feeding: soluble phenolics (total and individual), trypsin, and chymotrypsin inhibitors. Analyses were run for both treatments, EAB damaged (induced) and no damage (control), for all species examined above. The preparation of EAB feeding was as described in the preceding experiment. Mature leaves with (a branch from "induced treatment") and without (a branch from "control treatment") EAB feeding damage were cut and flash frozen in liquid nitrogen approximately 4 week after seedling potting. Samples then were ground to a fine powder with a mortar and pestle in liquid nitrogen.

Extraction of Soluble Phenolics Phenolics were extracted according to Eyles et al. 2007. Briefly, ~80–120 mg of homogenized foliage tissue were extracted twice overnight in the dark at 4 C with 500 μl of 100% MeOH. Extracts were pooled and transferred into a 1.5-ml microcentrifuge tube and centrifuged (12,000xg for 5 min) to remove solids. Samples were stored at -20 C and used in subsequent HPLC analyses.

Analysis and Identification of Soluble Phenolics HPLC-ESI-MS (Varian 500 MS; Palo Alto, CA, USA) in parallel with a PDA detector (Varian ProStar 335) were employed to detect and identify phenolics. Chromatographic separation of phenolics was carried out using a Waters Xterra™ RP18 analytical column (3.9 μm). The binary mobile phase consisted of water/acetic acid (99.9:0.1 v/v) (eluant A) and methanol/acetic acid (99.9:0.1 v/v) (eluant B) with a flow rate of 1 ml/min. The gradient was as follows (percentages refer to proportions of eluant B): 5–15% (0–15 min); 15–30% (15–35 min); 30–40% (35–40 min); 40–60% (40–50 min); 60–90% (50–55 min); 90–100% (55–60 min). The injection volume for all samples was 10 μl. Compound separation was achieved by post-column splitting (1:1), and passing the LC effluent through the PDA Detector (scanning range, 200–400 nm) and an electrospray source (parameters below). The ESI was in negative ion mode. The MS detector was optimized to obtain maximum yields of $[M-H]^-$ ions of apigenin, luteolin, rutin, oleuropein, tyrosol, and verbascoside standards. The optimized MS parameters were: capillary voltage, -80 V; needle voltage, -5 kV. The atmospheric pressure ionization (API) parameters were as follows: API nebulizing gas (air) 25 psi; API drying gas (nitrogen) 15 psi at 350 C. Survey scan was set to detect molecules between 50–1,000 m/z. Each treatment was run using both the TurboDDS scan type in the enhanced scan mode and full scan mode. The TurboDDS trigger threshold was set to 20,000 counts (parent ion counts). In the TurboDDS scan mode, MS scan parameters were set on-the-fly by the instrument to detect the most abundant parent ions. If TurboDDS was triggered, the mode temporarily switched

to MSn mode to perform daughter scans of the putative parent ions. The TurboDDS trigger threshold for daughter ions was 20,000, 2,000, and 200 ions for MS2, MS3, and MS4, respectively. Full scan parameters were set following the conditions mentioned above. Full scan chromatograms were overlaid with the PDA chromatogram traces at 280 nm to match $[M-H]^-$ parent ions to λ_{max} of individual phenolics. PDA data of individual compounds were analyzed using PolyView software (Varian). Phenolics were identified based on the congruence of parent and daughter ions, λ_{max}, and order of time compared to the published literature (Eyles et al. 2007). Data acquisition and processing were performed using MS Workstation 6 (Varian).

Quantitation and Analysis of Soluble Phenolics with HPLC-UV Quantitation of identified phenolics was performed using HPLC-UV. HPLC-UV analyses were performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler and a 996 PDA (Waters). The autosampler and column temperatures were set to 4 and 30 C, respectively. The binary mobile phase consisted of water/acetic acid (98:2, v/v) (eluant A) and methanol/acetic acid (98:2, v/v) (eluant B), with a flow rate of 1 ml/min. The elution program matched the conditions used for HPLC-ESI-MS-PDA analysis mentioned above. The injection volume for all samples was 10 µl. Samples were scanned in the range 200–400 nm. Individual compounds were quantified in mg g^{-1} FW. The identity of hydroxytyrosol, verbascoside, oleuropein, rutin, apigenin, and luteolin were unequivocally confirmed by spiking samples with the respective standards and matching retention times and UV maxima. Due to a lack of external standards for all compounds, samples were quantified following the method of Eyles et al. 2007. Compounds were expressed as hydroxytyrosol equivalents for hydroxytyrosol hexoside and tyrosol hexoside, verbascoside equivalents for verbascoside hexoside, oleuropein equivalents for elenolic acid derivative, ligustroside, nuzhenide, and oleuropein aglycone, apigenin equivalents for apigenin rutinoside, and luteolin equivalents for kaempherol rutinoside and kaempherol rhamnosylhexoside. Quantitation of individual compounds was carried out by comparing peak areas at 280 nm against a standard curve of the equivalent external standard. Total peak areas were taken to represent total phenolics (Bonello and Blodgett 2003; Blodgett et al. 2005). Total and individual peak areas at 280 nm for all species and treatment combinations were normalized to 100 mg dry weight.

Trypsin and Chymotrypsin Inhibitor Extraction and Analysis Trypsin and chymotrypsin inhibitors were extracted with a 10 mM Tris buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), a solution modified from Chen et al. (2007). The mixture was centrifuged at 10,000 g for 10 min. The supernatant was used for assay. The trypsin and chymotrypsin inhibitor assays followed Walsh and Wilcox (1970), and Broadway (1993) using *N*α-*p*-tosyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrates for trypsin and chymotrypsin determination, respectively. Each treatment was replicated 8 times.

Statistical Analysis Concentrations of volatile and non-volatile foliar compounds were compared individually between treatments using independent *t*-tests. Concentrations of volatiles and phenolics were square root transformed to homogenize variance, while no transformation was necessary for protease inhibitor data. All analyses were conducted in SAS with α=0.05 (SAS Institute 1999).

Results

Foliar Volatile Compound Responses to EAB Adult Feeding Induction of foliage volatile compounds by EAB adult feeding in black, green, and white ash is shown in Table 1. The most abundant volatiles were the three green leaf volatiles, hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, and a sesquiterpene, *Z,E*-α-farnesene, irrespective of species. Release of indole and benzyl cyanide by black ash was significantly induced, over 7-fold. Emanation of indole and (*E*)-β-ocimene (monoterpene) of green ash was elevated, while two green ash leaf volatiles, hexanal and (*E*)-2-hexenal, decreased. Release of seven volatiles of white ash, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, cubenene, (*E*)-β-ocimene, indole, benzyl cyanide, increased. The magnitude of increase ranged from 5-fold [(*Z,E*)-α-farnesene] to over 100-fold (benzyl cyanide). No other compound of the three species was significantly affected by EAB adult feeding.

Foliar Non-volatile Compound Responses to EAB Adult Feeding HPLC-UV chromatographic profiles of control (undamaged) black, green, and white ash are shown in Fig. 1. Tentatively identified and characterized phenolics are presented in Table 2. Only qualitative differences among species were detected (Tables 2, 3). A total of six, 13, and 10 phenolics were detected in black, green, and white ash, respectively. Among those, ligustroside was the only one detected in all three species. Oleuropein aglycone and 2 unidentified compounds (peak # 2 and 4) were found

Table 1 Volatile compound concentrations (mean±SE ng g^{-1} dry foliage tissue) of three ash species with undamaged leaves (constitutive) and leaves induced by emerald ash borer (EAB) adult feeding. N=8 in all cases

Compound	Black ash		Green ash		White ash	
	Constitutive	Induced[a]	Constitutive	Induced[a]	Constitutive	Induced[a]
1. Hexanal	609.3±63.1	464.5±82.2	1,118.6±140.0	718.3±126.2*	3,703.5±955.9	2,304.8±416.9
2. (E)-2-hexenal	160.0±14.3	134.9±22.0	281.9±33.7	185.4±29.0*	927.6±243.6	579.7±98.2
3. (Z)-3-hexenyl acetate	502.1±214.1	214.8±49.9	54.2±13.9	42.9±10.3	118.0±40.0	474.2±204.7
4. Z,E-α-farnesene	498.6±76.8	506.6±53.8	49.3±13.6	71.0±8.9	25.6±11.3	134.0±47.4**
5. α-humulene	5.64±1.3	6.5±2.6	0.8±0.2	2.1±0.7	4.6±1.0	33.3±13.2*
6. β-caryophyllene	31.4±6.4	35.2±12.2	2.4±0.7	7.0±2.4	19.0±5.30	167.5±61.0**
7. cubenene	47.2±13.0	78.5±33.1	14.5±4.8	22.3±11.8	10.5±2.4	79.0±33.0*
8. (E)-β-ocimene	77.1±17.1	133.3±41.1	7.6±1.1	16.0±2.2**	14.3±3.5	121.9±49.2*
9. Indole	3.6±0.5	25.3±8.9*	2.6±1.2	9.3±2.3**	2.1±1.0	21.2±8.1*
10. Benzyl cyanide	38.6±10.3	254.6±60.4**	1.7±0.7	3.9±1.1	0.5±0.2	63.6±30.3**
11. Nonanal	1.2±0.2	1.5±0.2	3.6±0.7	2.9±0.5	4.4±1.2	5.2±0.9

[a] Ash foliage was fed upon by 10, 8-10-d old EAB female adults for 48 h; * and ** denote significant difference at α=0.05 and 0.01 between control and EAB treatments within the same ash species, respectively

only in black ash; coumaroylquinic acid and feruloylquinic acid were detected only in green ash; and verbascoside hexoside was detected only in white ash (Tables 2, 3). EAB adult feeding did not elicit or decrease concentrations of any of a number of selected compounds (most prominent peaks in their respective chromatograms,,Table 3). The levels of total foliar phenolics in white ash decreased significantly upon EAB feeding but were unaffected in black and green ash foliage (Fig. 2).

EAB adult feeding did not affect production of trypsin inhibitor in any species (Fig. 3a). Neither was production of chymotrypsin inhibitor in green or white ash changed (Fig. 3b). Chymotrypsin inhibitor concentration of EAB adult-fed black ash foliage was greater than that of undamaged control (Fig. 3b).

Discussion

The most abundant constitutive volatiles from all three species were three green leaf volatiles [hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate] and a sesquiterpene (*Z,E*-α-farnesene). Indole was induced by EAB adult feeding in all three species. However, in previous work, indole did not elicit any electro-antennal activities of EAB females or males (Rodriguez-Saona et al. 2006).

Other volatiles detected from foliage responded differentially to EAB adult feeding. Headspace concentrations of hexanal and (*E*)-2-hexenal of black and white ash were not affected by EAB adult feeding, while they were suppressed in green ash. Both the reduction of these two compounds and the effect on EAB need further investigation because EAB males and females have been shown to be responsive to hexanal and (*E*)-2-hexenal in GC-EAD bioassays (Rodriguez-Saona et al. 2006; de Groot et al. 2008). However, neither of these two compounds seemed to be attractive to EAB males or females under field conditions (de Groot et al. 2008).

The monoterpene (*E*)-β-ocimene was among the most commonly released volatiles following herbivore damage. Its emission was not elevated in black ash, while it increased significantly in green and white ash. Many herbivores, including the sphinx moth *Manduca sexta* (Lepidoptera: Noctuidae) (Fraser et al. 2003) and EAB (Rodriguez-Saona et al. 2006) have been observed to respond to (*E*)-β-ocimene in EAD bioassays.

Elevated levels of four volatiles, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene, were induced by EAB feeding in white ash. These compounds have been suggested to be involved in host plant location by many herbivores and host location by some parasitoids (Dicke et al. 1990; Meiners et al. 2003). They have been found to be antennally-active in EAB (Rodriguez-Saona et al. 2006; Crook et al. 2008), and the latter three are components of *Manuka* oil which is attractive to EAB in the field (Crook et al. 2008).

Benzyl cyanide emission was elevated 6-fold by EAB feeding in black ash and 100-fold in white ash, while it was not affected in green ash. Benzyl cyanide is produced by both plants (Bartlet et al. 1997, Rodriguez-Saona et al. 2006) and insects (Seidelmann et al. 2000, Fatouros et al. 2008), and it has been hypothesized to have many ecological functions in intraspecific and interspecific interactions. For instance,

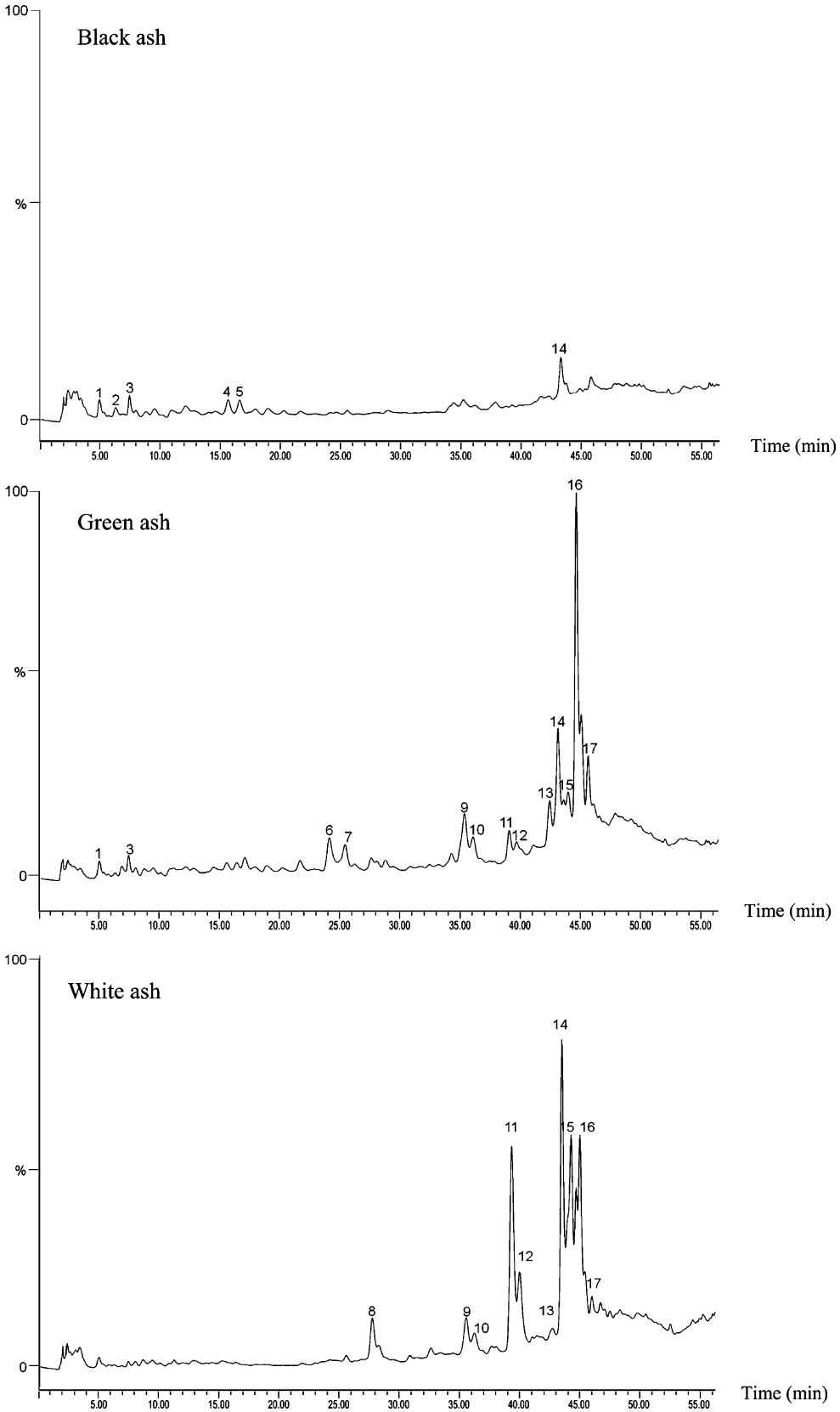

Fig. 1 HPLC-UV chromatographic profiles at 280 nm of methanol extracts of black, green, and white ash control foliage. Numbers above peaks refer to compounds listed in Table 2

Table 2 Tentative identification of phenolics from black, green and white ash based on retention time, *m/z*, MS fragmentation, and UV absorbance maxima

Peak Number	Species[a]	RT (min)	$[M-H]^-$ (*m/z*)	Fragments *m/z* (in order of decreasing abundance)	UV (λ) maxima (nm)	Putative ID	Reference
1	G, B	5.03	315	153, 123	278.9	Hydroxytyrosol hexoside	Cardoso et al. 2005
2	B	6.38	ND[b]	ND	360.3, 271.8	Unknown #1	N/A
3	G, B	7.51	299	113, 119, 143, 179	276.5	Tyrosol hexoside	Kammerer et al. 2005
4	B	15.82	ND	ND	312.1	Unknown #2	N/A
5	B	16.62	377	197, 153, 109	280.1, sh[c] 316	Oleuropein Aglycone	Cardoso et al. 2005
6	G	24.41	337	191, 163, 173, 127, 85	sh 290, 312.1	Coumaroylquinic Acid	Poon 1998
7	G	25.61	367	ND	280.1, 333.5	Feruloylquinic Acid	Bastos et al. 2007
8	W	27.79	785	623, 461, 315	sh 285, 331.1	Verbascoside hexoside	Eyles et al. 2007
9	G, W	35.52	623	461, 315, 135	sh 285, 331.1	Verbascoside	Cardoso et al. 2005
10	G, W	36.13	685	523, 453, 421, 299, 153, 179	280.1, 331.1	Nuzhenide	Ryan et al. 2002
11	G, W	39.29	569	537, 403, 223, 357,121	281.2	Oleuropein related compound	Cardoso et al. 2005, Savarese et al. 2007
12	G, W	39.77	539	377, 307, 275, 139	281.2	Oleuropein	Tanahashi et al. 1998
13	G, W	42.62	609	301, 271, 179, 151	265.9, 349.0	Rutin	Savarese et al. 2007
14	G, W, B	43.35	523	361, 291, 259, 111, 139	sh 280	Ligustroside	Ryan et al. 2002
15	G, W	44.23	577	269, 225, 197	267.0, 339.5	Apigenin-7-*O*-rutinoside	Plazonić et al. 2009
16	G, W	44.86	593	285, 255, 211, 227	268.2, sh 290, 338.3	Kaempferol-O-coumaroyl hexoside	Gouveia and Castilho 2010
17	G, W	45.89	593	285, 257	265.9, sh 290, 350.2	Luteolin-7-*O*-rutinoside	Plazonić et al. 2009

[a] B: black ash; G: green ash; W: white ash

[b] ND = Not detected

[c] Shoulder

benzyl cyanide released from oilseed rape (*Brassica napus* L.) serves as an attractant for cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) (Bartlet et al. 1997). In insects, male-originated benzyl cyanide functions as an anti-aphrodisiac for females and kairomones for *Trichogramma brassicae* (Hymenoptera:Trichogrammatidae), the egg parasitoid of *P. brassicae* females (Fatouros et al. 2005). However, benzyl cyanide was not among the volatile compounds in Manchurian ash that elicited electroantennal activity in EAB adult males or females (Rodriguez-Saona et al. 2006).

Three noteworthy points concerning volatile ash emissions in this study are: (1) A lack of antennal or behavioral response by EAB to significantly induced volatiles such as indole does not necessarily negate an effect on other organisms such as predators and parasitoids of EAB. Attraction of herbivore-induced plant volatiles to natural enemies of herbivores is well documented (Heil 2008; Chen et al. 2010); (2) Although not significantly elevated, levels of (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene in EAB fed seedlings of green and black ash were numerically higher than those in respective control seedlings. The effects of potential increases of these compounds on attraction of EAB requires further investigation; (3) The effects of changes in ratios of these compounds deserve further examination because not only trap placement, trap size and color, but also ratios and release rates of VOCs, are important in trapping and detection of EAB (Crook and Mastro 2010).

Table 3 Concentrations (mean±1SE mg g^{-1} fresh weight) of selected individual phenolic compounds in green, white, and black ash

Peak #[a]	Black ash		Green ash		White ash	
	Constitutive (*N*=8)	Induced[a] (*N*=8)	Constitutive (*N*=8)	Induced (*N*=8)	Constitutive (*N*=7)	Induced (*N*=7)
1	$6.5\times10^{-2}\pm1.4\times10^{-2}$	$5.9\times10^{-2}\pm1.3\times10^{-2}$	$6.4\times10^{-2}\pm0.6\times10^{-2}$	$5.7\times10^{-2}\pm0.7\times10^{-2}$	ND	ND
3	$4.6\times10^{-2}\pm0.3\times10^{-2}$	$5.3\times10^{-2}\pm0.8\times10^{-2}$	$0.5\times10^{-1}\pm0.1\times10^{-1}$	$3.2\times10^{-2}\pm0.4\times10^{-2}$	ND	ND
5	$25.6\times10^{-2}\pm5.2\times10^{-2}$	$40.5\times10^{-2}\pm13.5\times10^{-2}$	ND	ND	ND	ND
8	ND[b]	ND	ND	ND	$26.8\times10^{-2}\pm6.1\times10^{-2}$	$12.8\times10^{-2}\pm2.5\times10^{-2}$
9	ND	ND	$49.1\times10^{-2}\pm12.7\times10^{-2}$	$5.7\times10^{-1}\pm1.6\times10^{-1}$	$47.5\times10^{-2}\pm9.0\times10^{-2}$	$50.9\times10^{-2}\pm2.8\times10^{-2}$
10	ND	ND	$58.7\times10^{-2}\pm1.6$	$3.2\times10^{-1}\pm.18\times10^{-1}$	$74.3\times10^{-2}\pm12.8\times10^{-2}$	70×10^{-2} 3±8.3$\times10^{-2}$
11	ND	ND	$62.2\times10^{-2}\pm16.5\times10^{-2}$	$39.5\times10^{-2}\pm2.2\times10^{-2}$	4.6±1.1	2.5±0.9
12	ND	ND	$38.9\times10^{-2}\pm9.5\times10^{-2}$	$40.6\times10^{-2}\pm7.8\times10^{-1}$	3.6±0.8	1.8±0.7
13	ND	ND	$32.4\times10^{-2}\pm5.1\times10^{-2}$	$40.4\times10^{-2}\pm7.7\times10^{-2}$	$13.5\times10^{-2}\pm4.5\times10^{-2}$	$13.8\times10^{-2}\pm6.5\times10^{-2}$
14	0.6±0.1	0.4	1.6±0.5	0.3±0.3	4.4±1.7	2.0±0.5
15	ND	ND	$8.3\times10^{-2}\pm3.6\times10^{-2}$	$33.5\times10^{-2}\pm3.0\times10^{-2}$	$39.7\times10^{-2}\pm10.9\times10^{-2}$	$27.6\times10^{-2}\pm0.2\times10^{-2}$
16	ND	ND	$51.1\times10^{-2}\pm7.7\times10^{-2}$	$75.5\times10^{-2}\pm15.7\times10^{-2}$	$37.1\times10^{-2}\pm7.7\times10^{-2}$	$22.3\times10^{-2}\pm1.9\times10^{-2}$
17	ND	ND	$21.4\times10^{-2}\pm6.2\times10^{-2}$	$7.3\times10^{-2}\pm1.8\times10^{-2}$	$7.9\times10^{-2}\pm2.9\times10^{-2}$	$7.8\times10^{-2}\pm4.8\times10^{-2}$

No significant differences for each individual phenolic compound were found from the same ash species between control and induced foliage

[a] See Table 2 for tentative identification; Ash foliage was fed upon by 10, 8-10-d old EAB female adults for 48 h

[b] ND=Not detected

Phenolics generally denature plant proteins and thus reduce nutritive values of plants (Dudt and Shure 1994; Ossipov et al. 2001). Phenolics also can inhibit the absorption of key amino acids. For example, cyclochampedol, a flavonoid from acetone bark extracts of the tree *Arctocarpus champeden* (Thunb.) Merr., inhibits absorption of leucine in the midgut of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) (Parenti et al. 1998). Significant qualitative differences among the three ash species were observed. Qualitative difference of phloem phenolics between the Asian ash species, Manchurian ash, and two North American species, green and white ash, have been noted previously (Eyles et al. 2007). Our study shows also, however, that compared to phloem, ash foliage appears to contain fewer phenolic compounds, regardless of ash species.

EAB adult feeding did not induce accumulation of individual selected foliar phenolics in the present study, irrespective of species, perhaps due to the nutrient regime used. For example, many volatile and non-volatile compounds that are involved in cotton (*Gossypium hirsutum* cv. FiberMax 989) or Austrian pine (*Pinus nigra* Arnold) defense are suppressed by high nitrogen fertilization (Blodgett et al. 2005; Chen et al. 2008), and the nitrogen supply in the present study was relatively high. Second, the lack of induction likely was due to a phenomenon called "delayed induced resistance" (DIR) in which induction of defensive compounds occurs in the years following defoliation. Induced resistance can be either DIR or "rapidly induced resistance" (RIR) in which defensive compounds appear shortly after damage (Karban and Myers 1989). DIR has been observed in many plant species, in particular, in trees (Kaitaniemi et al. 1998). Third, the lack of induction might reflect a general pattern of the response of mature foliage to biotic and abiotic stress (Nichole-Orians 1991; McAuslane et al. 1997; Chen and Poland 2009b).

However, EAB adult feeding induced a decrease of total soluble phenolics in white ash (Fig. 1). This may partially explain why EAB adults consumed more white ash foliage given choices between black and white ash foliage (Chen and Poland 2010). Furthermore, the

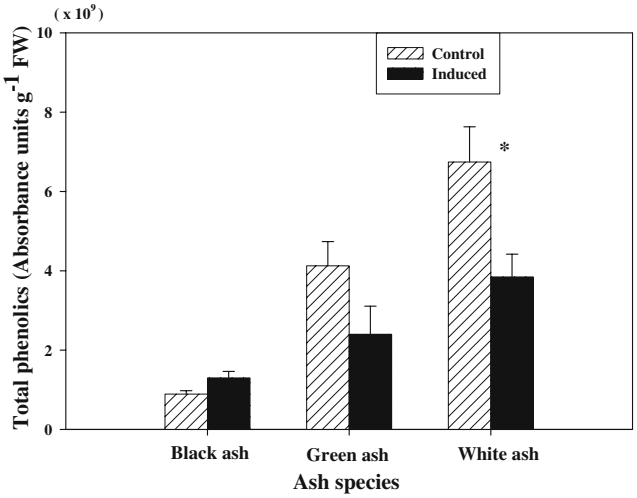

Fig. 2 Total soluble phenolics (mean±SE absorbance units g^{-1} fresh weight tissue) in control (undamaged) foliage and foliage induced by emerald ash borer (EAB) adult feeding of black, green, and white ash. Data were analyzed by independent *t*-test, separately for ash species. Significant differences within species are indicated by asterisks (*: $P<0.05$). Black ash: $N_{Control}=8$; $N_{EAB}=8$; Green ash: $N_{Control}=8$; $N_{EAB}=8$; White ash: $N_{Control}=7$; $N_{EAB}=7$

longevity of EAB adults was greater when fed white ash compared to black ash (Chen and Poland 2010). The three phenolics uniquely detected in black ash (oleuropein aglycone, unknowns #1 and 2; Table 2) might also cause EAB adults to prefer green and white ash over black ash (Chen and Poland 2010). Compared to green and white ash, black ash is phylogenetically more closely related to Manchurian ash (Wallander 2008), which has been observed to be more resistant to EAB infestation (Rebek et al. 2008).

Many protease inhibitors are involved in herbivore defense (Ryan 1990, Zavala et al. 2004). Protease inhibitors impair herbivore growth by eventual depletion of essential amino acids resulting from a herbivore's compensatory production of digestive proteases (Broadway and Duffey 1986). EAB feeding did not affect trypsin inhibitors of black, green, or white ash (Fig. 3a), or chymotrypsin inhibitors of green or white ash (Fig. 3b). The activity of chymotrypsin inhibitors, however, did increase in black ash as a result of EAB feeding (Fig. 3b), which, together with the unique phenolics in black ash discussed above, might contribute to the preference of EAB adults for green and white ash over black ash (Chen and Poland 2010).

In summary, EAB adult feeding differentially affected volatile and non-volatile compounds of the three most common ash species in northeastern USA. Black ash was the least responsive species in terms of volatile induction with only two (indole and benzyl cyanide) of eleven compounds responding. Green ash was more responsive than black ash, with two [(*E*)-β-ocimene and indole] of eleven volatiles induced while two green leaf volatiles [hexanal and (*E*)-2-hexenal] decreased. White ash showed the strongest response with an increase in seven of eleven compounds. Black and white ash seedlings were more responsive to EAB adult feeding than green ash in terms of two non-volatile, putatively defensive compound groups, i.e., phenolics and protease inhibitors. Phenolic production decreased in white ash while chymotrypsin increased in black ash. The induction of chymotrypsin inhibitors by EAB feeding, together with unique phenolics in black ash, might explain, in part, EAB adults' preference for green and white ash over black ash (Chen and Poland 2010). Future studies will focus on screening of potent compounds from ash foliar volatile and non-volatile compounds identified in this study and incorporating them into ash breeding programs to develop resistant trees.

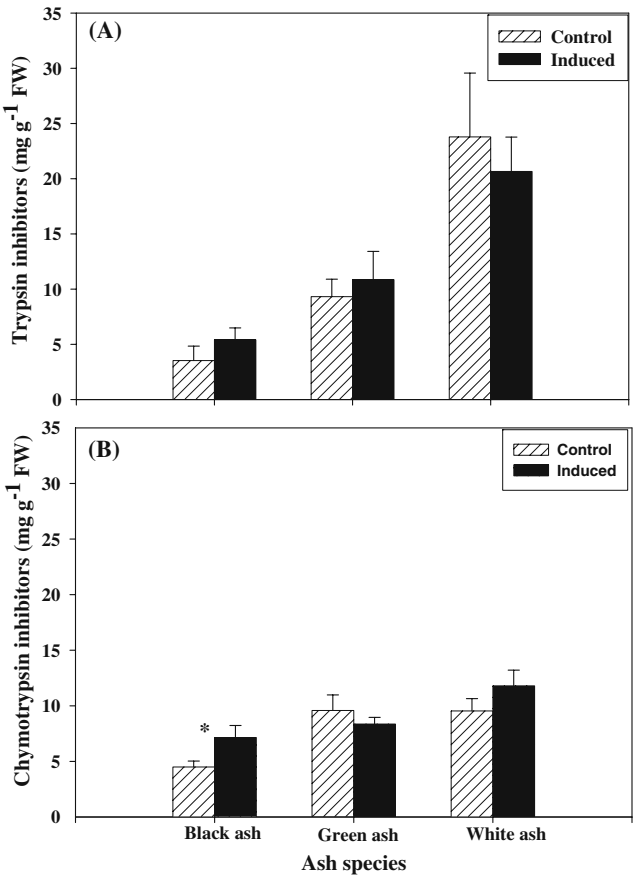

Fig. 3 Total concentrations (mean±1SE mg g^{-1} fresh weight tissue) of foliar trypsin **a** and chymotrypsin inhibitors **b** in control (undamaged) foliage and foliage induced by emerald ash borer (EAB) adult feeding of black, green, and white ash. Data were analyzed by independent *t*-test, separately for ash species. Significant differences within species are indicated by asterisks (*: $P<0.05$). Black ash: $N_{Control}=8$; $N_{EAB}=8$; Green ash: $N_{Control}=8$; $N_{EAB}=8$; White ash: $N_{Control}=8$; $N_{EAB}=8$

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