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Interspecific Comparison of Constitutive Ash Phloem Phenolic Chemistry Reveals Compounds Unique to Manchurian Ash, a Species Resistant to Emerald Ash Borer

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Abstract The emerald ash borer (*Agrilus planipennis*, EAB) is an invasive wood-borer indigenous to Asia and is responsible for widespread ash (*Fraxinus* spp.) mortality in the U.S. and Canada. Resistance and susceptibility to EAB varies among *Fraxinus* spp., which is a result of their co-evolutionary history with the pest. We characterized constitutive phenolic profiles and lignin levels in the phloem of green, white, black, blue, European, and Manchurian ash. Phloem was sampled twice during the growing season, coinciding with phenology of early and late instar EAB. We identified 66 metabolites that displayed a pattern of variation, which corresponded strongly with phylogeny. Previously identified lignans and lignan derivatives were confirmed to be unique to Manchurian ash, and may contribute to its high level of resistance to EAB. Other compounds that had been considered unique to Manchurian ash, including hydroxycoumarins and the phenylethanoids calceolarioside A and B, were detected in closely related, but susceptible species, and thus are unlikely to contribute to EAB resistance of Manchurian ash. The distinct phenolic profile of blue ash may contribute to its relatively high resistance to EAB.

Keywords *Agrilus planipennis* · *Fraxinus* · Wood-borer · HPLC · Host plant resistance · Plant–insect interactions · Invasive species · Emerald ash borer · Coleoptera · Buprestidae

Introduction

Emerald ash borer [(EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)] is an invasive wood-boring beetle indigenous to Asia that has caused widespread mortality

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of ash (*Fraxinus* spp.) trees in the U.S., Canada, and Russia (Poland and McCullough, 2006; Baranchikov et al., 2008; Smith et al., 2009). Larvae feed on the phloem and outer xylem, disrupting translocation of nutrients and water and ultimately causing tree death (Herms et al., 2004; Cappaert et al., 2005; Poland and McCullough, 2006). Black (*F. nigra* Marshall), white (*F. americana* L.), green (*F. pennsylvanica* Marshall), and European (*F. excelsior* L.) ash have all experienced extensive mortality, while in one study blue ash (*F. quadrangulata* Michaux.) was less extensively colonized than white ash, suggesting that it may be more resistant to EAB (Cappaert et al., 2005; Poland and McCullough, 2006; Anulewicz et al., 2007; Rebek et al., 2008). Observations in Asia suggest that Manchurian ash (*F. mandshurica* Ruprecht), which has coevolved with EAB, is not aggressively attacked unless stressed (Wei et al., 2004; Liu et al., 2007). These observations were confirmed experimentally in a common garden experiment where Manchurian ash was found to be much more resistant to EAB than green and white ash, supporting the hypothesis that Asian species possess defenses by virtue of their evolutionary history with EAB (Rebek et al., 2008).

Resistance of deciduous trees to wood-boring, phloem feeding insects is hypothesized to result from a combination of constitutive and induced physical and chemical defenses (Dunn et al., 1990; Eyles et al., 2007). The secondary chemistry of the genus *Fraxinus* is highly diverse (Kostova and Iossifova, 2007). Recently, it was noted that constitutive phenolic compounds, including hydroxycoumarins and phenylethanoid glycosides (calceolariosides A and B), were found in phloem tissue of Manchurian ash but not in green and white ash. These compounds were hypothesized to contribute to the high resistance of Manchurian ash to EAB (Eyles et al., 2007; Cipollini et al., 2011).

Identification of resistance mechanisms inferred from interspecific comparisons of resistant and susceptible species can be confounded by phylogenetic variation in traits that do not contribute to variation in host quality (Agrawal, 2011). Within the genus *Fraxinus*, green and white ash are phylogenetically distant from Manchurian ash, and some of the previously documented variation in their phenolic profiles (Eyles et al., 2007; Cipollini et al., 2011) may be the result of evolutionary divergence that is not related to their differences in resistance to EAB. Black and European ash are phylogenetically closely related to the resistant Manchurian ash, with all three species belonging to the section Fraxinus, but both of the former species are highly susceptible to EAB (Wallander, 2008). Variation in the phloem chemistry of these closely related resistant and suceptible species may have resulted from differential selection pressure imposed by EAB. Hence, we conducted an extensive metabolite profile characterization of interspecific variation in the constitutive phenolic chemistry of the phloem of ash species, with the prediction that closely related Manchurian, black, and European ash would be more similar to each other than to green, white, and blue ash, and that compounds unique to Manchurian ash might be related to its resistance against EAB. Trees were sampled twice during the growing season to coincide with the phenology of early and late instar EAB. We also quantified interspecific variation in lignin concentrations, a phenolic polymer that can also contribute to insect resistance (Coley, 1986; Wainhouse et al., 1990; Kurokawa et al., 2004; Borg-Karlson et al., 2006), but that showed little variation among a number of *Fraxinus* species in an earlier study (Cipollini et al., 2011).

Methods and Materials

Experimental Design and Sampling Ash trees were grown in an experimental plantation that included Manchurian ash cv. 'Mancana', green ash cv. 'Patmore', and white ash cv. 'Autumn Purple', all of which are clonally propagated cultivars. The plantation also included blue, green, and European ashes that were propagated as open-pollinated seedlings. The three cultivars and three open-pollinated species were planted in a common garden in November 2007 in Bowling Green, OH, USA in a randomized complete block design with eight blocks. Each of the six taxa was replicated four times within each block (4 individual tree replicates × 6 taxa = 24 trees per block). Thirty-two black ash cv. 'Fallgold' trees were planted in a plot adjacent to the original common garden plantation on 1 May 2008. On 2 June 2008, the mean stem diameter (measured 15 cm above ground) of Manchurian ash cv. 'Mancana', black ash cv. 'Fallgold', green ash cv. 'Patmore', white ash cv. 'Autumn Purple', and seedling-propagated green ash, blue ash, and European ash were 3.1± 0.06 cm (SEM), 2.6±0.06, 3.0±0.05, 3.0±0.1, 2.5±0.1, 1.7± 0.09, and 1.8±0.08, respectively. A previous study has shown that ash trees of this size, including some of the same cultivars, are readily colonized by EAB under field conditions in a common garden in Michigan, while exhibiting differences in resistance to EAB attack (Rebek et al., 2008), with Manchurian ash cv. 'Mancana' clearly being resistant. The relative resistance of Manchurian ash cv. 'Mancana' to EAB attack was confirmed in a follow-up to this study in which our common garden was exposed to augmented populations of the insect in the year following this investigation (Whitehill, 2011).

Trees were sampled at each of two time points, 2 June and 6 August 2008, with one tree for each taxon per block randomly selected for the sampling. All samples were from unique trees except for one case in which the same white ash tree was sampled and analyzed in both June and August. Phloem tissue was sampled for phenolic analyses on those dates to correspond phenologically with the beginning of egg hatch and the presence of mature larvae (3 rd and 4th

instars), respectively (Cappaert et al., 2005). Two-yr-old branches were pruned from trees, placed on ice and transported to the lab where phloem tissue was excised immediately from the branch, frozen in liquid nitrogen, and stored at −80 °C until sample extraction.

Phenolic and Lignin Extraction Soluble phenolics were extracted according to Eyles et al. (2007) and Cipollini et al. (2011). Extracts were stored at −20° C and used in subsequent HPLC analyses within 3 wk following extraction. Lignin was extracted and measured according to Bonello and Blodgett (2003).

Analysis of Phenolic Compounds HPLC-UV analyses of phenolic extracts were performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler and a 996 Photodiode Array Detector (PDA). The autosampler and column temperatures were set to 4 and 30 °C, respectively. Chromatographic separations were accomplished using a Waters Xterra™ RP18, 5 μm, 4.6×150 mm column and a Waters Xterra™ RP18, 3.9 μm, 3.0×20 mm guard column. The binary mobile phase consisted of water/acetic acid (A) (98:2, *v*/*v*) and methanol/acetic acid (B) (98:2, *v*/*v*), with a flow rate of 1 ml/min. The elution program followed that of Eyles et al. (2007). The injection volume for all samples was 10 μl. Samples were passed through the PDA (scanning range, 200–400 nm) with individual peaks quantified at 280 nm. The absorbance limit for detection was set to a minimum peak area of 9,000 in the processing parameters prior to data export. Individual peaks were included in subsequent statistical analyses if they met the following criteria: 1) a peak height greater than 0.02 AU, 2) a clearly discernible UV spectrum that could be matched to known phenolic compounds and/or could be interpreted in conjunction with MS data, and 3) consistent detection in at least three individual trees within a taxon.

For each sampling date, phenolic extracts from each taxon also were analyzed using an HPLC-ESI-MS (Varian 500 MS; Palo Alto, CA, USA) in parallel with a PDA detector. For detailed methods used in the qualitative identification of phenolic compounds see Supplemental Materials and Methods.

Individual compounds were quantified following the methods of Eyles et al. (2007). Matching standards were used for compound quantification when available. In cases where no matching standard was available, standard curves generated by using related compounds were used for relative quantification. For detailed methods used in compound quantification see Supplemental Materials and Methods.

Statistical Analyses Principal component analysis (PCA) was used to investigate the overall relationships between phenolic metabolites and species through dimensionality reduction and feature extraction (Johnson and Wichern, 2002). The PCA was conducted using R software (R Development Core Team, 2011) for both species and metabolites. PCA was run twice in order to better visualize relationships between species. The second PCA was based on data obtained from a cluster analysis of species and metabolites following the first PCA. Cluster analysis was run to support the grouping of variables obtained in the PCA. The cluster analyses were carried out using the "pvclust" routine in the pvclust package in R (Suzuki and Shimodaira, 2006). The pvclust package generates approximately unbiased (AU) confidence values for the clusters using the bootstrap resampling technique to assess their reliability (Efron et al., 1996; Suzuki and Shimodaira, 2006). A total of 1,000 bootstrap replications were generated for each cluster, and the AU confidence values were used to assess the uncertainty. The significance level of the clusters was set to 95. A larger confidence value is indicative of a "true" cluster (Efron et al., 1996). In this study, we also used biplots constructed with R to identify the relationship between species and metabolites.

Individual peak areas and mg g^{-1} FW concentrations for lignin were compared among species using univariate analysis of variance (ANOVA). Exploratory analyses of data and Levene's test were used to evaluate variance equality and normality requirements of residuals. Square-root and logarithmic transformations were used to meet normality requirements of residuals and homogeneity of variance. Following significant *F*-tests, means were separated using the LSD test (α=0.05). All data were analyzed with IBM SPSS Statistics v. 19 (SPSS Inc., 2010).

Results

A total of 66 individual phenolic compounds (Figs. 1, 2 and 3) were selected for analysis from all seven taxa, including the clonally and seedling-propagated green ash, from the combined June (Table S1) and August (Table 1) datasets. Only August data are presented in the paper (Tables 1 and 2), while the June data are presented in supplemental materials (Tables S1 and S2). Classes of phenolic compounds that we found in ash phloem include simple phenolics, phenolic acids, hydroxycoumarins, lignans, secoiridoids, phenylethanoids, flavonoids, and coumarin-secoiridoids. These compounds are similar to the phenolic metabolites previously described for the genus *Fraxinus* (Eyles et al., 2007; Kostova and Iossifova, 2007; Cipollini et al., 2011). Qualitative patterns of phloem chemistry among the taxa reflected their phylogenetic relationships, and quantities of phenolic compounds tended to increase from June to August (Tables 1 and 2; Figs. 1, 2, 3 and 4; Tables S1 and S2; and Fig. S1, S2, S3, S4 and S5). Detailed information regarding fragmentation patterns of phenolic metabolites identified in this study and data analysis,

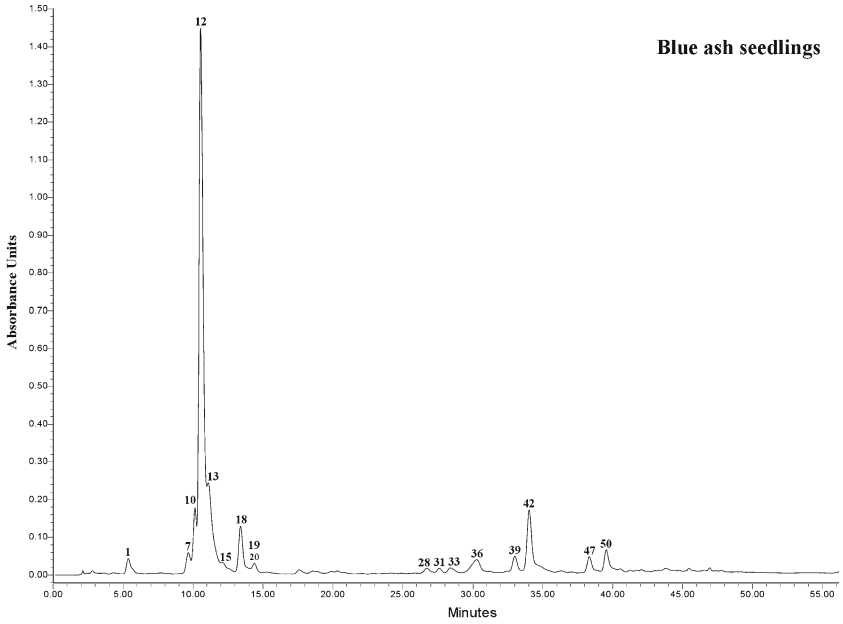

Fig. 1 Representative HPLC chromatogram at 280 nm of phloem tissue extracts from blue ash seedlings—a member of the section Dipetalae (Wallander, 2008). The chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

interpretation, and presentation of qualitative and quantitative differences in phenolic profiles can be found in Supplemental Results.

Lignin Lignin concentrations tended to be higher in August than in June and were consistently higher in phloem of blue ash and black ash cv. 'Fallgold' than in the other species (Tables 2 and S2). European ash had the lowest concentration of lignin in both June and August (Tables 2 and S2). White ash cv. 'Autumn Purple' and Manchurian ash cv. 'Mancana' had consistently higher lignin concentrations than green ash cv. 'Patmore' and seedling-propagated green ash.

Discussion

Manchurian ash cv. 'Mancana' is much more resistant to EAB than North American green ash cv. 'Patmore' or white ash cv. Autumn Purple', perhaps due to targeted selection imposed by its co-evolutionary history with EAB (Liu et al., 2007; Rebek et al., 2008; Whitehill, 2011). Earlier characterization of the constitutive phenolics in the phloem of these three cultivars revealed several compounds unique to Manchurian ash, including several hydroxycoumarins and two phenylethanoid compounds, calceolariosides A and B. These compounds were hypothesized to contribute to Manchurian ash resistance to EAB (Eyles et al., 2007; Cipollini et al., 2011). However, we detected hydroxycoumarins and calceolariosides A and B in the constitutive phloem of susceptible black and European ash in quantities comparable to or greater than those found in Manchurian ash, contradicting previous studies that were focused on differences between Manchurian, green, and white ash (Eyles et al., 2007; Cipollini et al., 2011). Of the ash species and cultivars we compared in this study, the qualitative phenolic profile of Manchurian ash cv. 'Mancana' differed most from green ash cv. 'Patmore' and white ash cv. 'Autumn Purple', which makes the comparison of these species the least informative when attempting to identify compounds potentially involved in resistance. This pattern is consistent with their distant phylogenetic relationship (Figs. 1, 2, 3 and 4) (Wallander, 2008). While some of the variation in the phenolic chemistry of Manchurian, white, and green ash may contribute to their differential resistance to EAB, much of their phytochemical variation may be the result of evolutionary divergence unrelated to selection imposed by EAB.

Black and European ash, which are highly susceptible to EAB, belong to the same section (Fraxinus) as Manchurian ash, and thus provide a phylogenetically-controlled comparison for identifying compounds that might be involved in EAB resistance . We found that phloem of black ash cv. 'Fallgold' and European ash contained a diverse array of hydroxycoumarins. Given their presence in the highly susceptible black and European ash, it is unlikely that these compounds play an important role in resistance of Manchurian ash to EAB. A role for hydroxycoumarins in resistance to EAB (Eyles et al., 2007) is not supported by our data.

Eyles et al. (2007) also hypothesized that calceolarioside A and B contributed to Manchurian ash resistance to EAB. However, we found ca. three-fold higher concentrations of calceolarioside A in black ash cv. 'Fallgold', and two-fold higher concentrations of calceolarioside B in European ash, than in Manchurian

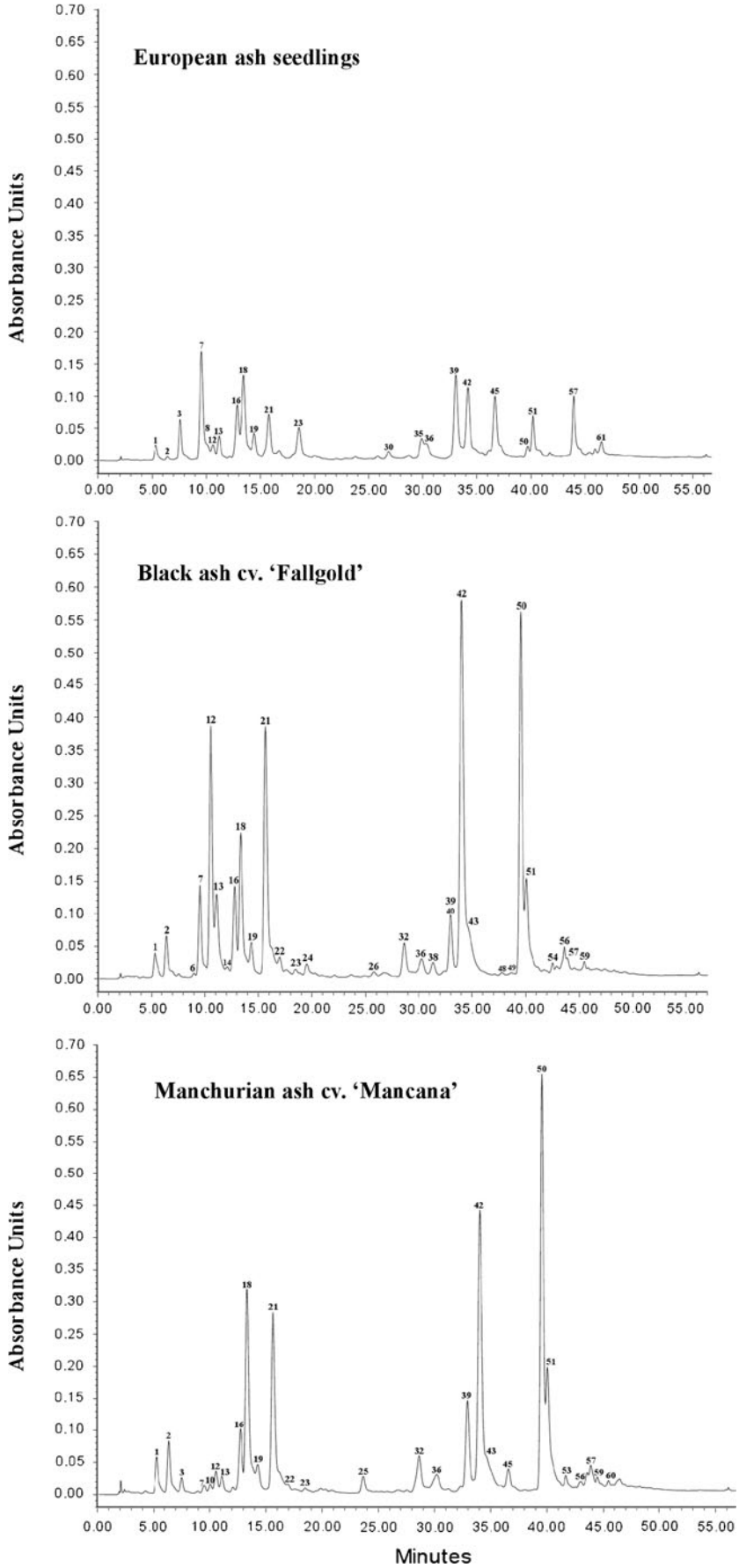

Fig. 2 Representative HPLC chromatograms at 280 nm of phloem tissue extracts from European ash seedlings, black ash cv. 'Fallgold', and Manchurian ash cv. 'Mancana'—all members of the section Fraxinus (Wallander, 2008). Each chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

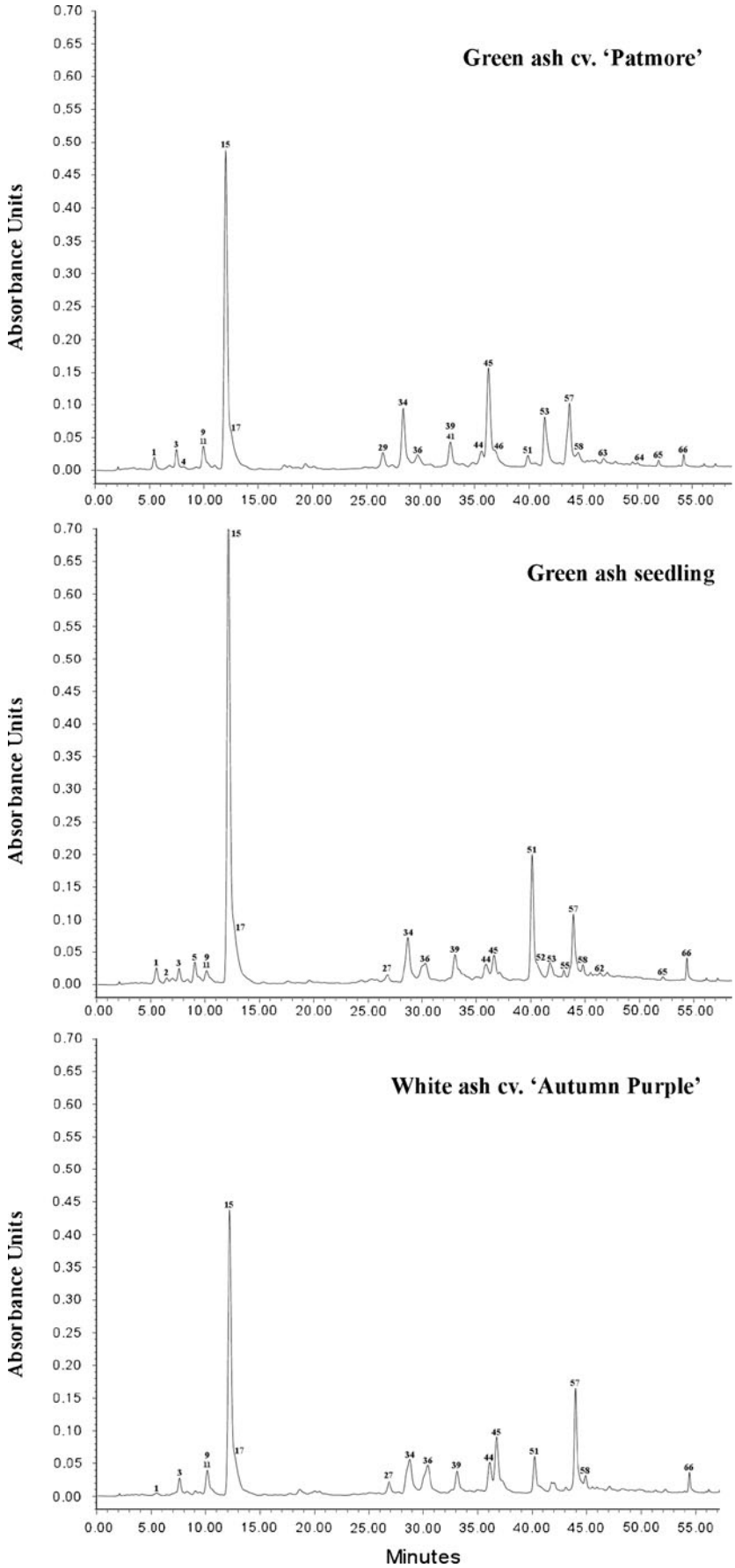

Fig. 3 Representative HPLC chromatograms at 280 nm of phloem tissue extracts from green ash cv. 'Patmore', green ash seedlings, and white ash cv. 'Autumn Purple'—all members of the section Melioides (Wallander, 2008). Each chromatogram represents a pool consisting of equal aliquots (100 μl each) of phloem extract from eight individual trees

Table 1 Phenolic compounds identified by HPLC-UV and LC-MS-PDA from samples collected on August 8th, 2008 from susceptible white ash cv. 'Autumn Purple', green ash cv. 'Patmore', green ash seedling, blue ash seedling, European ash seedling, black ash cv. 'Fallgold', and the resistant Manchurian ash cv. 'Mancana' grown in a common garden in Bowling Green, OH

Master Peak Number[a]	Species in which peak detected[b]	RT-HPLC-UV[c]	[M-H]- or [M-H]+[d*]	Fragments m/z (in order of decreasing abundance)[e]	λ max (nm)[f]	Putative ID[g]	Reference[h]
1	B, Q, E, GS, M, W	5.22	315	MS2: 135, 179; MS3: 107, 91, 117, 93, 79	281.2	Hydroxytyrosol hexoside	Cardoso et al., 2005; Eyles et al., 2007
2	B, GS, E, M	6.2	389	MS2: 329, 167, 161	276.5	Vanillic acid hexoside acetate adduct A	Eyles et al., 2007; Jimenez et al., 2010
3	GS, E, W, G, M	7.37	299	MS2: 179, 119, 143, 113, 161, 131	275.3	Tyrosol hexoside	Kammerer et al., 2005; Eyles et al., 2007
5	GS	8.59	389	MS2: 329, 167, 161	280.1	Vanillic acid hexoside acetate adduct B	Eyles et al., 2007; Jimenez et al., 2010
7	B, Q, E, M	9.24	177	MS2: 133; MS3: 89	289.5, 340.7	Esculetin A	Eyles et al., 2007
9	G, GS, W	9.76	601	MS2: 403, 223; MS3: 223, 371, 179; MS4: 121	264.7, sh[i] 300	Elenolic acid derivative 1 (m/z 431; 275 UV max)	Eyles et al., 2007
10	M, Q	10.02	177	MS2: 133; MS3: 89	258.8, sh 300, 340	Esculetin B	Eyles et al., 2007
12	B, Q, E, M	10.22	339	MS2: 177; MS3: 133, 105, 89, 149	334.7, sh 295	Esculin	Parejo et al., 2004; Eyles et al., 2007
13	B, Q, E, M	10.77	177	MS2: 133; MS3: 89	339.5, 289.5, 258	Esculetin C	Eyles et al., 2007
14	B	11.38	ND-LC-MS	ND-LC-MS	334.7, sh 293, 257.6	Unknown Coumarin 1	–
15	GS, W, Q, G	11.8	395*	MS2: 233, 185; MS3: 217, 203	264.7	Syringin	Eyles et al., 2007; Kostova and Iossifova, 2007
16	B, E, M	12.44	223*	MS2: 208, 163, 190, 107; MS3: 190, 180; MS4: 162, 134	289.5, 341.9	Fraxidin A	Yasuda et al., 2006
18	B, Q, E, M	12.99	369	MS2: 207; MS3: 192; MS4: 108, 164, 120, 175	341.9, sh 300	Fraxin	Godecke et al., 2005; Eyles et al., 2007
19	B, E, M	13.94	223*	MS2: 208, 163, 190, 107; MS3: 190, 180; MS4: 162, 134	293.1, 339.5	Fraxidin B	Yasuda et al., 2006
20	Q	13.94	177	MS2: 133; MS3: 89	345.4, 298, sh 260	Esculetin	Eyles et al., 2007
21	B, E, M	15.25	385*	MS2: 223; MS3: 208, 190; MS4: 190	328.7	Mandshurin	Terazawa and Sasaya, 1970; Eyles et al., 2007
22	B	16.17	209*	MS2: 149, 163, 181, 194; MS3: 121; MS4: 93, 65	339.5	Fraxetin	Eyles et al., 2007
23	B, E	17.64	353	MS2: 191, 179, 161	326.4, sh 298	3-Caffeoyl-quinic acid (Chlorogenic acid)	Cardoso et al., 2005
24	B	18.71	573	MS2: 537, 375; MS3: 375; MS4: 179, 327	277.7	Unknown Lignoid 1	–
25	M	23.64	717	MS2: 519, 357; MS3: 357; MS4: 151, 136	276.5	Pinoresinol dihexoside + $2H_2O$	Terazawa and Sasaya, 1970; Eyles et al., 2007
27	G, GS	26.66	523	MS2: 361; MS3: 165, 179, 313; MS4: 147	278.9	Ligustroside A	De la Torre-Carbot et al., 2005
28	Q	26.31	571	MS2: 373, 535, 343; MS3: 343; MS4: 285, 313, 207	281.2	(+)-1-Hydroxypinoresinol-4′-*O*-glucoside + $2H_2O$	Guo et al., 2007
29	W	26.44	525	MS2: 363, 301, 345; MS3: 301, 181, 199, 283; MS4: 283	278.9	Oleuropein Related Compound 1	Cardoso et al., 2005; Eyles et al., 2007
31	Q	27.24	ND by LC-MS PDA	ND by LC-MS PDA	278.9	Unknown Secoiridoid 1	–
32	B, M	27.69	477	MS2: 315, 203, 179, 341, 397; MS3: 135; MS4: 107	326.4, sh 290	Calceolarioside C	Eyles et al., 2007
33	Q	28.02	523	MS2: 361; MS3: 165, 179, 313	278.9	Ligustroside B	Ryan et al., 2002
34	G, GS, W	28.08	785	MS2: 623; MS3: 477, 461, 315; MS4: 315	331.1, sh 290	Forsythoside A *O*-glucoside	Guo et al., 2007
35	E	28.9	335	MS2: 179, 135 MS3: 135 MS4: 107, 117, 91, 79	327.6, 295	Caffeoylshikimic acid	Fang et al., 2002; Lin and Hamly, 2008
36	B, Q, E, G, GS, M, W	29.73	555		sh 280	10-Hydroxyoleuropein	

Table 1 (continued)

Master Peak Number[a]	Species in which peak detected[b]	RT-HPLC-UV[c]	[M-H]- or [M-H]+[d*]	Fragments m/z (in order of decreasing abundance)[e]	λ max (nm)[f]	Putative ID[g]	Reference[h]
				MS2: 393, 273, 307, 361, 375, 357, 419; MS3: 273, 307, 357, 343, 361; MS4: 137			Hosny, 1998; Kostova and Iossifova, 2007
38	B	30.43	525	MS2: 481, 195, 389, 319, 345; MS3: 345; MS4: 165	281.2	Dimethyoleuropein	Savarese et al., 2007
39	B, Q, E, G, GS, M, W	32.5	357	MS2: 151, 136, 311; MS3: 136; MS4: 92, 108	278.9	Pinoresinol	Eyles et al., 2007
42	B, Q, E, M	33.5	477	MS2: 315, 323, 179, 203, 341; MS3: 135 MS4: 107	328.7, sh 290	Calceolarioside A	Eyles et al., 2007
44	G, W	35.55	539	MS2: 377, 275, 291, 359; MS3: 275, 291, 359, 179; MS4: 111, 155	sh 280	Oleuropein A	Eyles et al., 2007
45	E, G, GS, M, W	36.04	623	MS2: 461; MS3: 315, 297; MS4: 135	331.1, sh 290	Verbascoside	Eyles et al., 2007
46	W	36.72	623	MS2: 461; MS3: 315, 297; MS4: 136	281.2, 331.1	Verbascoside A	Eyles et al., 2007
47	Q	37.89	725	MS2: 339; MS3: 177; MS4: 133	335.9, sh 295	Escuside	Iossifova et al., 2002;
50	B, E, Q, M	39.11	477	MS2: 315, 281, 251, 179, 221; MS3: 135; MS4: 107	328.7, sh 290	Calceolarioside B	Eyles et al., 2007
51	B, E, G, GS, M, W	39.68	539	MS2: 377, 307, 275; MS3: 307, 275, 345; MS4: 275, 139	281.2	Oleuropein	Cardoso et al., 2005; Eyles et al., 2007
52	GS	41.27	793.9	MS2: 403, 589, 743, 748, 595; MS3: 223, 371	265	Elenolic acid derivative 2	Eyles et al., 2007
53	GS, M, W	41.75	623	MS2: 461; MS3: 315, 297; MS4: 135	327.6, sh 288	Verbascoside B	Eyles et al., 2007
54	B	42.02	545	MS2: 369; MS3: 207, 192; MS4: 192	329.9, sh 295	Fraxin Related Compound	Godecke et al., 2005; Eyles et al., 2007
56	B, M	43.09	553	MS2: 391, 321, 289; MS3: 321, 289, 223; MS4: 139, 171, 167, 143, 261	280.1	Oleuropein Related Compound 2	Tanahashi et al., 1996; Hosny, 1998
57	B, E, G, GS, M, W	43.6	523	MS2: 361; MS3: 291, 259; MS4: 111, 139, 171, 143, 259	sh 280	Ligustroside	Tanahashi et al., 1996; Eyles et al., 2007
58	G, GS, W	44.36	431	MS2: 269; MS3: 225, 197, 117, 151	339.5, 268.2	Apigenin glucoside	Ryan et al., 2002
59	B, M	44.77	501	No fragmentation data available	328.7, sh 295	Unknown Coumarin 2	–
60	M	45.48	ND by LC-MS PDA	ND by LC-MS PDA	327.6, sh 298	Unknown Coumarin 3	–
61	E	46.01	447	MS2: 285, 255, 327, 227; MS3: 255, 227; MS4: 227, 211	350, sh 295, 264	Kaempherol galactoside	Ye et al., 2005
62	GS	46.03	ND LC-MS PDA	ND LC-MS PDA	sh 280	Unknown Secoiridoid 2	–
63	W	46.86	ND LC-MS	ND LC-MS	322.8, sh 282	Unknown Phenylethanoid 1	–
65	GS, W	51.82	285	MS2: 175, 199, 217, 241, 243, 151; MS3: 147, 131, 133, 119, 146; MS4: 119	254, sh 267, sh 290, 349.0	Luteolin	Ryan et al., 1999
66	G, GS, W	54.19	269	MS2: 225, 149, 151, 201; MS3: 181, 183, 197; MS4: 155, 141, 154	339.5, sh 290, 268.2	Apigenin	Eyles et al., 2007

[a] Number corresponding to a single individual peak identified using HPLC-UV. Peak numbers were assigned according to their retention time

[b] Letters correspond to the species in which the compound was detected via HPLC-UV (*B* black; *E* European; *G* green; *GS* green seedling; *Q* blue; *M* Manchurian; *W* white)

[c] RT-HPLC-UV = the average retention time for all individual biological replicates for each compound from all species

[d] The dominant molecular ion $[M-H]^-$ detected via LC-MS. Compounds were run in parallel with a PDA detector and full scan and PDA chromatograms were overlaid in order to match mass data. In some instances a $[M-H]^-$ could not be detected, in which case the positive ion mode was utilized to detect and identify the compound under investigation. For compounds identified using the positive ion mode, the corresponding $[M-H]^+$ is denoted with an *

[e] Corresponding fragmentation of the dominant molecular ion. Mass spectra for MS2, MS3, and MS4 are shown where applicable. Fragments are ordered according to decreasing abundance, with bolded ions representing the main ions fragmented in subsequent fragmentations

[f] λ max associated with each compound

[g] Tentative compound identity assigned based on literature match

[h] Corresponding reference for which a compound has been previously described

[i] *sh* = shoulder

Table 2 Contents of individual phenolic compounds and lignin in susceptible white ash cv. 'Autumn Purple', green ash cv. 'Patmore', green ash seedling, blue ash seedling, European ash seedling, black ash cv. 'Fallgold', and the resistant Manchurian ash cv. 'Mancana' in samples collected on August 8th, 2008. Compounds are separated into groups by phenolic compound class, while species are separated into the sections to which they belong within the genus *Fraxinus* (Wallander, 2008). Contents are expressed in mg g^{-1} FW±SEM (n=8). Different letters within a row indicate significantly different means by the protected LSD test (α=0.05). Black ash data appear in bold and are separate from the other species because it was not part of the original experimental design, but can be visually compared to the other species

Peak #	Compound name	*Melioides*			*Dipetalae*	*Fraxinus*		
		White	Green 'Patmore'	Green Seedling	Blue	European	Manchurian	Black
Phenolic acids/simple phenolics								
1	Hydroxytyrosol hexoside	0.4±0.03 b	ND[a]	0.4±0.1 b	1.1±0.1 a	0.5±0.1 b	1.2±0.1 a	**1.0±0.04**
2	Vanillic acid hexoside acetate adduct A	ND	ND	0.1±0.02 b	ND	0.1±0.01 b	1.0±0.04 a	**0.8±0.1**
3	Tyrosol hexoside	0.6±0.1 b	0.5±0.04 bc	0.4±0.1 c	ND	1.2±0.2 a	0.5±0.05 bc	**ND**
5	Vanillic acid hexoside acetate adduct B	ND	ND	0.4±0.1	ND	ND	ND	**ND**
23	3-Caffeoyl-quinic acid (Chlorogenic acid)	ND	ND	ND	ND	1.2±0.4	ND	**0.2±0.03**
Coumarins								
7	Esculetin A	ND	ND	ND	0.4±0.1 b	1.2±0.3 a	$0.1±3e^{-3}$ c	**0.9±0.1**
10	Esculetin B	ND	ND	ND	0.4±0.2 a	ND	$0.1±3e^{-3}$ b	**ND**
12	Esculin	ND	ND	ND	63.0±5.0 a	1.0±0.2 c	1.3±0.1 b	**15.0±0.3**
13	Esculetin C	ND	ND	ND	1.5±0.3 a	0.2±0.1 b	0.2±0.01 b	**1.0±0.1**
14	Unknown Coumarin 1	ND	ND	ND	ND	ND	ND	**0.1±0.04**
16	Fraxidin A	ND	ND	ND	ND	1.6±0.3 a	1.3±0.2 a	**2.0±0.2**
18	Fraxin	ND	ND	ND	8.7±0.9 b	8.6±1.7 b	22.5±1.6 a	**16.0±0.6**
19	Fraxidin B	ND	ND	ND	ND	0.7±0.1 a	0.7±0.1 a	**0.9±0.1**
20	Esculetin	ND	ND	ND	0.2±ND	ND	ND	**ND**
21	Mandshurin	ND	ND	ND	ND	1.1±0.1 b	5.0±0.5 a	**6.9±0.8**
22	Fraxetin	ND	ND	ND	ND	ND	ND	**1.6±0.5**
54	Fraxin Related Compound	ND	ND	ND	ND	ND	ND	**0.7±0.1**
59	Unknown Coumarin 2	ND	ND	ND	ND	ND	1.1±0.1	**1.2±0.1**
60	Unknown Coumarin 3	ND	ND	ND	ND	ND	0.7±0.1	**ND**
Monolignol								
15	Syringin	7.3±1.2 b	7.6±0.6 a	11.4±1.4 a	0.6±0.4 c	ND	ND	**ND**
Lignans								
24	Unknown Lignoid 1	ND	ND	ND	ND	ND	ND	**1.6±0.2**
25	Pinoresinol dihexoside + $2H_2O$	ND	ND	ND	ND	ND	1.7±0.3	**ND**
28	(+)-1-Hydroxypinoresinol-4′-*O*-glucoside + $2H_2O$	ND	ND	ND	1.3±0.4	ND	ND	**ND**
39	Pinoresinol	3.5±0.7 b	2.7±0.3 b	2.5±0.4 b	3.4±0.6 b	9.5±1.7 a	10.1±0.7 a	**7.0±0.4**
Phenylethanoids								
32	Calceolarioside C	ND	ND	ND	ND	ND	3.7±0.4	**3.0±0.2**
34	Forsythoside A *O*-glucoside	7.2±2.6 a	3.9±0.2 a	3.8±0.6 a	ND	ND	ND	**ND**
42	Calceolarioside A	ND	ND	ND	10.1±1.4 b	6.5±1.6 b	26.3±1.8 a	**34.6±1.1**
45	Verbascoside	4.3±1.2 a	4.2±0.3 a	1.7±0.2 b	ND	5.0±1.4 a	1.7±0.2 b	**ND**
46	Verbascoside A	1.2±0.3	ND	ND	ND	ND	ND	**ND**
50	Calceolarioside B	ND	ND	ND	3.0±1.4 b	0.7±0.2 c	25.2±2.6 a	**21.2±1.3**
53	Verbascoside B	3.9±0.5 a	ND	0.3±0.1 c	ND	ND	1.1±0.1 b	**ND**
63	Unknown Phenylethanoid 1	0.4±0.1	ND	ND	ND	ND	ND	**ND**

Table 2 (continued)

Peak #	Compound name	*Melioides*			*Dipetalae*	*Fraxinus*		
		White	Green 'Patmore'	Green Seedling	Blue	European	Manchurian	Black
Secoiridoids								
27	Ligustroside A	ND	2.5±0.2 a	1.6±0.3 b	ND	ND	ND	**ND**
29	Oleuropein Related Compound 1	3.0±0.4	ND	ND	ND	ND	ND	**ND**
31	Unknown Secoiridoid 1	ND	ND	ND	1.7±0.2	ND	ND	**ND**
33	Ligustroside B	ND	ND	ND	2.6±0.3	ND	ND	**ND**
36	10-Hydroxyoleuropein	3.4±0.3 d	9.4±0.8 a	5.2±0.5 c	7.5±0.9 b	4.1±0.6 cd	5.4±0.3 c	**4.5±0.2**
38	Dimethyoleuropein	ND	ND	ND	ND	ND	ND	**3.0±0.5**
44	Oleuropein A	3.2±0.2 b	5.4±0.7 a	ND	ND	ND	ND	**ND**
51	Oleuropein	1.8±0.1 d	5.1±0.5 c	19.2±2.7 b	ND	6.9±0.8 c	26.2±2.1 a	**20.7±2.0**
56	Oleuropein Related Compound 2	ND	ND	ND	ND	ND	1.3±0.1	**4.0±0.5**
57	Ligustroside	10.4±1.7 b	14.8±1.2 a	9.8±1.1 b	ND	8.1±1.2 b	3.9±0.4 c	**1.4±0.5**
62	Unknown Secoiridoid 2	ND	ND	0.8±0.3	ND	ND	ND	**ND**
Coumarin-Secoiridoid								
47	Escuside	ND	ND	ND	2.1±0.2	ND	ND	**ND**
Flavonoids								
58	Apigenin glucoside	0.2±0.01 ab	0.2±0.02 a	0.1±0.02 b	ND	ND	ND	**ND**
61	Kaempherol galactoside	ND	ND	ND	ND	0.3±0.1	ND	**ND**
65	Luteolin	$0.1\pm8e^{-3}$ a	ND	$0.04\pm6e^{-3}$ b	ND	ND	ND	**ND**
66	Apigenin	$0.1\pm6e^{-3}$ b	0.1±0.01 a	0.1±0.01 a	ND	ND	ND	**ND**
Phenolic polymer								
	Lignin	18.5±1.0 b	14.6±1.2 c	13.6±1.0 c	21.6±0.8 a	5.8±0.8 d	18.1±1.2 b	**20.5±1.0**

[a] *ND* = not detected

ash cv. 'Mancana'. The high concentrations of these compounds in black and European ash make it unlikely that calceolariosides are involved in Manchurian ash resistance to EAB.

Of the 27 phenolic compounds that we identified in the phloem tissue of Manchurian ash cv. 'Mancana', only pinoresinol dihexoside **(25)**, and a compound tentatively identified as a coumarin derivative **(60)**, were unique to this cultivar. Because the other 25 compounds were also detected in susceptible ash species, they are unlikely to contribute to EAB resistance, unless they act synergistically with compounds unique to Manchurian ash.

Eyles et al. (2007) detected high concentrations of pinoresinol dihexoside in dormant tissue of Manchurian ash relative to green and white ash and suggested that it may play a role in EAB resistance. We confirmed that concentrations of this compound remained high during the growing season on dates highly relevant to the phenology of EAB larvae, as did Cipollini et al. (2011). Pinoresinol dihexoside is a lignan (phenylpropane dimer) (Strack, 1997), and its aglycone (pinoresinol) has antifeedant and growth/molt inhibiting activities against several insect species (Miyazawa et al., 1994; Cabral et al., 2000; Garcia et al., 2000). A recent proteomic analysis of ash phloem showed that a phenylcoumaran benzylic ether reductase (PCBER) is expressed constitutively at >25-fold higher levels in phloem of Manchurian ash cv. 'Mancana' than in black ash cv. 'Fallgold', green ash cv. 'Patmore', and white ash cv. 'Autumn Purple' (Whitehill et al., 2011). PCBER is an enzyme involved in the biosynthesis of lignans, providing further support for a potential role of lignans in resistance of Manchurian ash cv. 'Mancana' to EAB.

Our inability to identify conclusively the coumarin derivative **(60)** precludes informed speculation about its putative role in EAB resistance. The functions of this compound could be tested by bioassays with EAB larvae in a phloem-free artificial diet (Keena et al., 2010), with compounds tested at biologically relevant concentrations (Tables 2 and S2).

Lignin can act as an indirect chemical defense (Borg-Karlson et al., 2006), or as a dose-dependent physical defense against wood-boring insects (Wainhouse et al., 1990). However, we found little evidence that variation in lignin concentration of phloem contributes to interspecific variation in EAB resistance, confirming earlier observations by

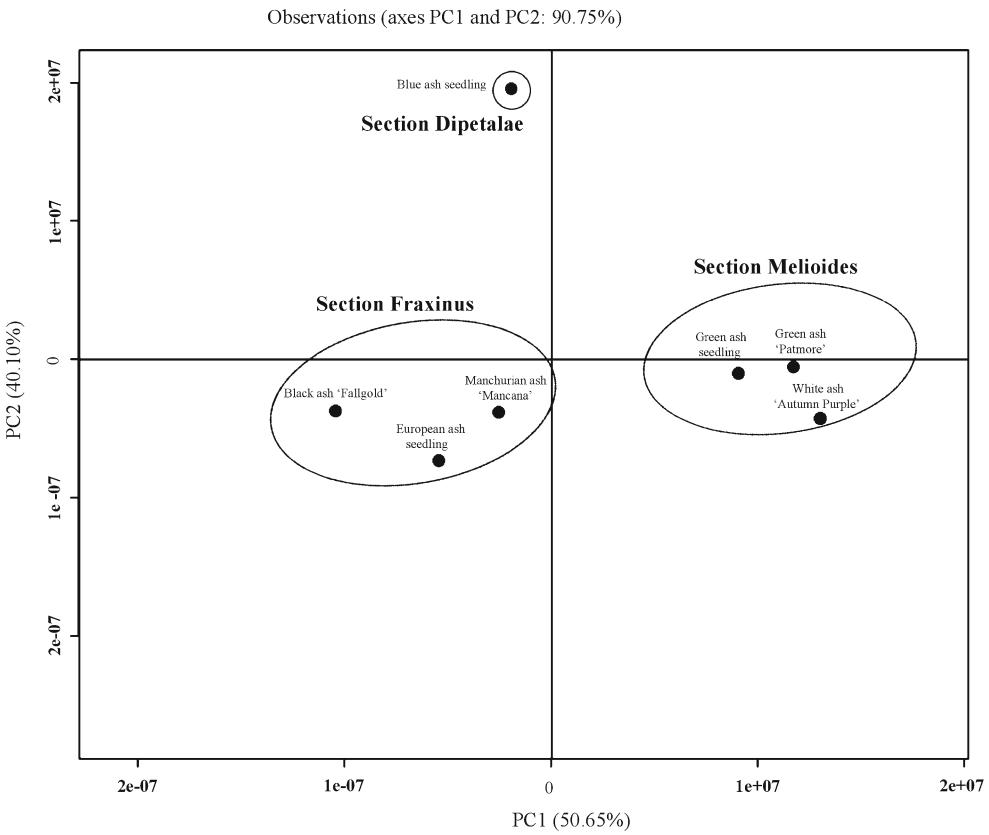

Fig. 4 Relationship between PC1 and PC2 scores for individual peak areas for 59 compounds, but excluding the most characteristic compounds (**12**, **15**, **18**, **21**, **42**, **50**, and **51**) that were associated with individual ash species (see Figs. 1, 2 and 3). Black dots represent the mean of eight biological replicates within a given taxon. The taxa cluster according to phylogenetic placement into the three groups Meliodes (green and white ash), Dipetalae (blue ash), and Fraxinus (Manchurian, European, and black ash)

Cipollini et al. (2011). Lignin concentrations in phloem of Manchurian ash cv. 'Mancana' were similar to those in white ash cv. 'Autumn Purple' and lower than in black ash cv. 'Fallgold', which are both highly susceptible. The high levels of lignin in blue ash were similar to the levels found in the highly susceptible black ash cv. 'Fallgold.'

Four of the taxa (black ash cv. 'Fallgold', green ash cv. 'Patmore', white ash cv. 'Autumn Purple', and Manchurian ash cv. 'Mancana') that we compared were clonally propagated cultivars, and thus their chemistry may not be representative of their respective species as a whole. However, their relative resistance to EAB is well characterized, so they provide relevant samples of these taxa to compare in the search for EAB resistance mechanisms. The other three taxa that we examined (blue, European, and green ash) were propagated as open-pollinated seedlings. We observed extremely high phytochemical similarity between the green ash clone and the seedling-propagated green ash population, as did Cipollini et al. (2011). We also observed high convergence in the phenolic profiles between species in the same taxonomic section (Fig. 4).

Blue ash, which is endemic to North America, has been colonized by EAB at lower levels than other North American species in the field (Anulewicz et al., 2007) and in a common garden study (Herms et al., unpublished). The very distinct phenolic chemistry of blue ash that we observed relative to that of green, white, and black ash cultivars may contribute to this resistance. Hence, blue ash may be a source of allopatric resistance (e.g., Harris and Frederiksen, 1984) that could be introgressed into the more susceptible North American congeners. However, the highly divergent phylogenetic relationships among these taxa (Jeandroz et al., 1997; Wallander, 2008) could complicate efforts to hybridize blue ash with more susceptible species.

The identification of resistant green and white ash genotypes could be important for host plant resistance breeding programs. A small proportion of 'lingering' green and white ash continue to survive in areas of high EAB-induced ash mortality, but further work is needed to establish their level of genetic resistance and to identify markers that could be used in programs to screen or breed for resistance (Koch et al., 2010). Pinoresinol dihexoside or unknown coumarin **(60)** may represent two of such biomarkers. Indeed, pinoresinol, the aglycone of pinoresinol dihexoside, was detected in all species we investigated. If a resistance role for pinoresinol is confirmed, the requisite genetic machinery for its production exists in susceptible North American ash species.

In summary, our broad-based interspecific survey of the phenolic profiles of ash phloem has confirmed that previously identified lignans and lignan derivatives may contribute to the high level of resistance of Manchurian ash cv. 'Mancana' to EAB. However, functional studies and/or bioassays are required to confirm the role of these compounds as deterrents or toxins for EAB larvae.

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