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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Decreased emergence of emerald ash borer from ash treated with methyl jasmonate is associated with induction of general defense traits and the toxic phenolic compound verbascoside

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Abstract The emerald ash borer (EAB; *Agrilus planipennis* Fairmaire) is causing widespread mortality of ash (*Fraxinus* spp.) in North America. To date, no mechanisms of host resistance have been identified against this pest. Methyl jasmonate was applied to susceptible North American and resistant Asian ash species to determine if it can elicit induced responses in bark that enhance resistance to EAB. In particular, phenolic compounds, lignin, and defense-related proteins were quantified, and compounds associated with resistance were subsequently tested directly against EAB larvae in bioassays with artificial diet. MeJA application decreased adult emergence in susceptible ash species, comparable to levels achieved by insecticide application. Concentration of the phenolic compound verbascoside sharply increased after MeJA application to green and white ash. When incorporated in an artificial diet, verbascoside decreased survival and growth of EAB neonates in a dose-dependent fashion. Lignin and trypsin inhibitors were also induced by MeJA, and analogs of both compounds reduced growth of EAB larvae in artificial diets. We conclude that the application of MeJA prior to EAB attack has the ability to enhance resistance of susceptible ash trees by inducing endogenous plant defenses, and report evidence that induction of verbascoside is a mechanism of resistance to EAB.

Keywords *Agrilus planipennis* · Biological invasions · *Fraxinus* · Induced resistance · Plant–herbivore interactions

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Introduction

The emerald ash borer [EAB; *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)] is an invasive wood-boring insect native to Asia that has killed millions of ash (*Fraxinus* spp.) trees since it was first discovered in southeastern Michigan in 2002 (Cappaert et al. 2005). EAB is a specialist of ash. Larvae feed on phloem, cambium, and outer xylem, girdling and eventually killing susceptible trees by disrupting translocation of water and nutrients throughout the main stem and branches (reviewed in Herms and McCullough 2014). Eastern North American ash species, including green (*F. pennsylvanica* Marshall), white (*F. americana* L.), black (*F. nigra* Marshall), and blue (*F. quadrangulata* Michx.) ash, are susceptible to attack by EAB to varying degrees (Rebek et al. 2008; Tanis and McCullough 2012; Klooster et al. 2014). Manchurian ash (*F. mandshurica* Ruprecht), which is native to Asia, is more resistant to EAB than most North American species, likely

through targeted defenses selected over time by virtue of its co-evolutionary history with the insect (Rebek et al. 2008).

Resistance mechanisms of deciduous trees against phloem-feeding buprestids are thought to include a combination of constitutive and induced chemical and physical defenses (Eyles et al. 2007; Muilenburg and Herms 2012). Thus, several studies have focused on identifying differences in constitutive proteomic and phytochemical profiles of bark tissues [consisting of the outer bark (periderm), cortex, phloem, cambium, and some secondary xylem] of susceptible North American and resistant Asian ash species (Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2011, 2012).

Plant responses to herbivory are often modulated by jasmonates, such as methyl jasmonate (MeJA) (Farmer and Ryan 1990; Gundlach et al. 1992; Howe and Jander 2008; Koo and Howe 2009). Hence, application of MeJA can prepare the plant to respond more quickly and forcefully to attack by an insect or pathogen (a priming effect) (Ballare 2011), or it may directly elicit induction of defense-related proteins and other phytochemicals (Erbilgin et al. 2006), a process we tested in this investigation.

Widespread ash mortality in urban environments is having a substantial economic impact (Kovacs et al. 2010). Insecticides, including soil- and trunk-applied systemic products and protective cover sprays applied to the bark such as bifenthrin, can effectively protect individual ash trees from EAB (Smitely et al. 2010; McCullough et al. 2011; Herms and McCullough 2014). However, induction of endogenous host-resistance mechanisms with defense elicitors represents a potential alternative to conventional insecticides. Plant elicitors, such as MeJA, can be patented, produced commercially, and applied to plants using conventional approaches (Farmer and Ryan 1990; Lyon et al. 1995). Yet the practical use of jasmonates to control pests has been limited in agriculture and forestry (Karban et al. 1997; Black et al. 2003; Rohwer and Erwin 2008). Jasmonates can decrease plant growth and crop yield by diverting resources towards defense (Cipollini and Heil 2010), although studies have demonstrated that proper timing and application method can reduce these problems when used in conjunction with an established integrated pest management plan (Thaler et al. 2002; Rohwer and Erwin 2008). Testing of jasmonates as tools for the actual management of forest pests has been limited, but studies have shown that they may be effective at controlling some tree pests under experimental conditions (Miller et al. 2005; Erbilgin et al. 2006; Zhao et al. 2011).

In this study, we examined the effect of MeJA application on EAB resistance and defensive chemistry of ash. We hypothesized that application of MeJA would protect susceptible ash species from EAB via induction of endogenous defenses (soluble phenolics, lignin, and defense proteins) in bark tissue; however, this effect would be less apparent in Asian ash species that are inherently more resistant to EAB. Finally, traits associated with resistance were tested directly against EAB larvae in an artificial diet.

Materials and methods

Experimental overview

The physiological effects of MeJA application on defense chemistry of *Fraxinus* spp. were quantified in separate experiments with containerized and field-grown ash plants. A MeJA induction study using containerized ash (conducted in 2008 and repeated in 2009 in the absence of EAB pressure) informed a subsequent field study in which ash trees planted in a common garden were treated with MeJA and subjected to uniform pressure from EAB.

Experiment 1: MeJA elicitation of containerized ash trees

Experimental design

Manchurian ash cv. 'Mancana' and white ash cv. 'Autumn Purple' were used in nursery experiments. Details regarding plant material details can be found in supporting information (Online Resource ESM 1). MeJA is a volatile molecule with the potential to induce biochemical changes in neighboring plants; therefore, we physically separated our trees into a MeJA-treated and a water-treated control group by placing the two groups on either side of a polyhouse, which separated the groups by approximately 15 m. In the first year of the experiment (2008), each group contained five white and four Manchurian ash. Trees were organized in a completely randomized design within each treatment group. Manchurian and white ash trees had mean stem diameters, measured 15 cm above the soil line, of 2.9 ± 0.2 cm (SE) and 3.0 ± 0.2 cm, respectively. Light intensity, temperature, and relative humidity were measured three times per day (1000, 1200, and 1400 hours) within each experimental group throughout the course of the experiment (11 days total) (Online Resource ESM 2).

We repeated the experiment in the summer of 2009 using the same ash varieties used in 2008. The MeJA and water treatment groups were separated as in the previous year, except the position of each treatment group was reversed (i.e., the location of the water treatment group in 2008 became the location of the MeJA treatment group in 2009 and vice versa). We also increased the number of biological replicates within each group to eight white and eight Manchurian ash trees in each control and MeJA-treated group. Trees were arranged in a completely randomized design within each group.

MeJA treatment and sample collection

MeJA or water was applied to a randomly selected branch from the lowest whorl on each tree. Branches in the MeJA treatment group received an exogenous application of a 1-M solution of MeJA (Sigma–Aldrich, St. Louis, USA; 95 % pure, w/w) in sterile water with 0.1 % Tween 20, while the second treatment group received only sterile water with 0.1 % Tween 20. A preliminary investigation had demonstrated that 1 M MeJA exogenously applied to ash outer bark could induce the accumulation of phenolics in bark tissues (data not shown). Lower concentrations of MeJA (0.1 M) had no significant effect on phenolic chemistry (Wang 2010), while higher concentrations (2 M) were phytotoxic, resulting in necrosis around lenticels located on the outer bark (personal observation).

A 20-cm segment of each selected branch was treated, beginning at the stem–branch junction, with 1 ml of either water or MeJA applied with a cotton swab until the bark surface was thoroughly wet. Treatments were applied every other day for a week corresponding to days 1, 3, 5, and 7 of the experiment. On day 11, treated branches were cut from trees at the stem junction, placed in Ziploc™ bags on ice, and transported back to the laboratory. There a 2-cm section of bark was excised, starting approximately 5 cm away from the stem–branch junction, immediately frozen in liquid N_2, and stored at −80 °C until phenolic and lignin extraction. The first experiment began on 2 July and concluded on 12 July 2008, while the second experiment began on 29 June and concluded on 9 July 2009, following the same treatment regime implemented in the first experiment. No significant differences in microclimate were observed in the summer of 2008 between the two treatment groups (Online Resource ESM 2), so environmental data were not acquired in the summer of 2009.

Experiment 2: common garden field experiment

Establishment and design of the common garden experiment

A common garden containing clonal individuals of Manchurian ash cv. ‘Mancana’, green ash cv. ‘Patmore’, and white ash cv. ‘Autumn Purple’, and seedlings of green ash was established in November 2007 in Bowling Green, OH. In April 2008 (one month after acquisition) black ash cv. ‘Fallgold’ trees were planted in a separate block adjacent to the common garden. Details regarding plant material can be found in supporting information (Online Resource ESM 1). In May 2008, all trees in the plot were sprayed with the insecticide bifenthrin (Onyx™, FMC, Philadelphia, PA, USA) at the highest labeled rate for wood-borers to prevent local populations of EAB from colonizing trees prior to initiation of experimental treatments. To protect trees from mammals and mechanical damage, all stems were wrapped with plastic tree guards, which were removed at the beginning of April 2009.

The experiment consisted of eight blocks with 12 trees per block for a total of 96 trees. The planting was designed as a randomized complete block (RCB) with Manchurian, green ‘Patmore’, white, and green ash seedlings each replicated three times per block. Trees were spaced 2.4 m apart within a block and blocks were spaced 3.6 m apart. A separate black ash block consisting of 24 trees was planted adjacent to the common garden following the same spacing parameters as the main block.

Experimental treatments

In 2009, three experimental treatments were assigned randomly to each taxon within a block, with each species × treatment combination replicated once within each of the eight blocks. For the black ash block, each treatment was randomly assigned to eight trees. Therefore, a total of 120 trees were used in the experiment (5 taxa × 3 treatments × 8 biological replicates). Trees received one of following three treatments: (1) 0.1 % Tween 20 in sterile water (control), (2) 1 M MeJA with 0.1 % Tween 20 in sterile water, and (3) bifenthrin (Onyx™) insecticide. Trees were treated once with insecticide at the labeled rate for wood-borers, during the third week of May, with the entire stem of each tree sprayed to runoff. MeJA was applied evenly along the main stem of each tree using a foam paintbrush on 27 May, 1 July, and 3 August 2009. The water control was applied in the same way as MeJA. When treatments were initiated on 27 May 2009, ash trees were 6 years old with mean stem diameters of 3.2 ± 0.06 cm (Manchurian), 3.2 ± 0.05 cm (green ‘Patmore), 2.8 ± 0.1 cm (green seedlings), 3.2 ± 0.1 cm (white), and 2.7 ± 0.08 cm (black), at 15 cm above the soil line. Trees of this size are large enough to be colonized by EAB (Rebek et al. 2008).

Augmentation of EAB adults in ash common garden

The common garden was planted in an area with high EAB population density, with the closest infested trees located ~0.5 km away. To homogenize EAB presence within the common garden, bolts harvested from ten heavily-infested green ash trees were placed in each block on 15 April 2009. Each tree was cut into ten bolts, which were then labeled 1–10 starting from the highest portion of the tree down to the base. Bolts were then randomized into nine groups of 11 and each group was assigned to a block. The bolts were ~60 cm long and had an average of 36.1 ± 3.3 (SE) exit holes/bolt at the end of the 2009 growing season. Thus, we estimate that we augmented the number of natural

immigrants from surrounding infested areas by more than 3,500 adults during the summer of 2009.

EAB emergence from common garden ash trees and analysis

Emergence holes of EAB adults were counted on 5 July 2010 by visually inspecting the main stem and branches of all trees. The 2008 insecticide spray did not completely prevent infestation, and the number of baseline EAB exit holes was recorded in the fall of 2009 for each tree. To evaluate the effectiveness of the treatments applied in the summer of 2009 on EAB attack rates and larval survival to adult emergence in 2010, we subtracted emergence holes counted in 2009 from emergence holes counted on the same trees in the fall of 2010 and defined the resulting variable as "exit holes per tree" formed in 2010. Emergence hole data for black ash were analyzed separately, since black ash trees were planted in an adjacent block that was not part of the initial RCB design. Emergence hole densities were normalized to "tree" because total bark surface area could not be calculated exactly on trunk and branches of small trees and all trees were very similar in size (i.e. no significant differences between stem diameters 15 cm above soil line of all species at start of experiment). Specific details relating to statistical analyses of emergence hole data can be found in supporting information (Online Resource ESM 1).

Tissue sampling and chemical analyses

Bark from second-year branches was used for all chemical analyses. Branches were removed from trees on 29 July 2009, stripped of leaves, placed on ice, and transported back to the laboratory where bark tissue was immediately removed, frozen in liquid N_2, and stored at −80 °C until sample extraction. Phenolics were extracted according to Eyles et al. (2007). Samples were then stored at −20 °C and used in subsequent HPLC analyses within 3 weeks following extraction. Lignin was extracted from pellets that remained following phenolic extraction according to the methods of Bonello et al. (2003). Individual concentrations (mg g^{-1} FW) of lignin were used for all statistical analyses. Specific details relating to statistical analyses of lignin data can be found in supporting information (Online Resource ESM 1).

HPLC-UV system specifications, methods for selection and quantification of phenolic compounds, and procedures for identification of phenolics are described in detail in Whitehill et al. (2012). Any deviations from published work, and details relating to statistical analyses, can be found in the supporting information (Online Resource ESM 1).

Soluble proteins were extracted and analyzed for total protein concentration, rate of browning, chitinase (CHI), peroxidase (POD), polyphenoloxidase (PPO), and trypsin inhibitor (TI) activity following the methods of Cipollini et al. (2011). Details relating to statistical analyses of defense proteins can be found in the supporting information (Online Resource ESM 1).

In vitro biossays using artificial diet

To examine the effect of putatively relevant chemical defenses on larval performance, we used a plant tissue-free artificial diet developed for EAB (Keena et al. 2009) and provided by the USDA Forest Service, Northeastern Center for Forest Health Research, Northern Research Station in Hamden, CT, USA. We tested the effects of verbascoside, trypsin inhibitor, and lignin, using commercially available sources. Concentrations tested were chosen to span the ranges observed in ash bark in both nursery and field grown trees (verbascoside and lignin) or field grown trees alone (trypsin inhibitors).

Verbascoside (>98 % purity; Alfa-Chemistry, Stony Brook, NY, USA) was dissolved in a dimethyl sulfoxide (DMSO; Fisher) solution and mixed by hand into a ground diet to achieve concentrations of 0, 5, 20.9 and 45 mg verbascoside g^{-1} diet. The DMSO concentration in the diet was held at 0.01 % (w/w). Soybean trypsin inhibitor (STI; Sigma) was dissolved in millipure water and mixed in the ground diet to achieve concentrations of 0, 4, 40, 400 mg STI protein g^{-1} diet. Preliminary studies using this protein at high levels resulted in complete mortality, so the upper concentration, while not ecologically relevant, was used to replicate this effect. Powdered spruce lignin (Sigma) was mixed directly into the ground diet to achieve concentrations of 0, 14, and 20 mg lignin g^{-1} diet, after which millipure water was added to moisten the diet. Diets were dried to approximately 50 % moisture content for 30–60 min in a fume hood. Approximately 24 h prior to the estimated larval eclosion date, ~1 g aliquots of each diet were packed into the bottom of individual 4-mL glass vials and capped. Each cap had a small hole for gas exchange. Verbascoside and lignin treatments had 15 replicates at each dose, while the STI treatments had 18 replicates at each dose. STI treatments were assayed at a separate time from the verbascoside and lignin treatments, using a different batch of larvae, but the same diet.

EAB eggs were acquired from a laboratory colony at the USDA APHIS PPQ Biocontrol Rearing Facility in Brighton, MI, USA. Eggs arrived in the laboratory no more than 2 days after oviposition. Immediately upon arrival, eggs were placed in cleaned plastic containers in an incubator set to 25 °C, with a 16:8 h (light:dark) cycle. A beaker of deionized water was placed in each container and kept

the relative humidity (RH) at 80 % (±5 %). A fine-tipped camel hair paintbrush was used to place neonates as they enclosed in a shallow etch that was scratched in the surface of the diet. Neonates were then lightly covered with crumbles of diet from the same vial. Vials were placed in the incubator in racks at 25 °C and 80 % RH (±5 %) in a closed, opaque plastic container for 30 days. Diets were supplemented with 200–300 µL of millipure water if the diet in a vial appeared to be desiccating.

After a 30-day incubation period, vials were removed from the incubator and examined under a dissecting microscope. Surviving larvae were extracted from the diet and weighed to the nearest 0.1 mg. In vials where no activity (feeding galleries or frass) was seen, diets were dissected under a dissecting scope in order to determine the fate of the larvae. If a larva was determined to have fed on the diet and died soon after, or to have been in a position to feed but had not accepted the diet and died, then it was designated as dead as a result of the treatment. Larvae that were determined to not have fed due to a mechanical inhibition (e.g., larva was accidentally placed in a position where it could not feed or could not move itself in a position to feed) were omitted from any analyses. This occurred infrequently and randomly across treatments. The proportion of larvae surviving in each treatment (for those treatments that had survivors) after the 30-day incubation was analyzed by logistic regression with compound dose as the predictor variable. Final mass of surviving larvae in each treatment was examined using linear regression with compound dose as the predictor variable. All statistical analyses of in vitro bioassay data were performed in R.

Results

Effect of MeJA treatment and species on individual phenolics and lignin of containerized manchurian and white ash trees (Experiment 1)

A total of 31 (2008) and 46 (2009) individual chromatographic peaks were analyzed from containerized Manchurian and white ash trees. Of the compounds selected for further investigation over the course of 2 years, concentrations of 17 were affected by treatment with MeJA in 2008 and 11 were affected in 2009 when both species were combined (Tables 1, 2; Table S1, Online Resource ESM 3). Only compounds that responded to MeJA induction are presented in this paper (Tables 1, 2; Table S1, Online Resource ESM 3) as constitutive compound information has been reported previously (Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2012). In white ash, tyrosol hexoside (2; Tables 1, 2) decreased in both years, and verbascoside (17; Tables 1, 2) concentration increased by 175 % in 2008 and 195 % in 2009 in response to MeJA (Table 2; Table S1, Online Resource ESM 3). Tyrosol hexoside (2) and verbascoside (17) were not affected by application of MeJA in Manchurian ash over the 2 years of experiments with containerized trees (Tables 2; Table S1, Online Resource ESM 3). Application of MeJA increased lignin concentration in both Manchurian and white ash in 2008, but the increase was only marginally significant in 2009 for both species ($P = 0.09$) (Tables 2; Table S1, Online Resource ESM 3).

In Manchurian ash, concentrations of vanillic acid hexoside (1; Table 2), esculetin C (5), fraxidin A (7), fraxin (8), fraxidin B (9), mandshurin (10), fraxetin (11), calceolarioside C (13), calceolarioside A (15), and calceolarioside B (19) all increased in 2008 (Tables 2 and S1). In 2009, concentrations of fraxidin A (7), unknown secoiridoid 1 (14), and oleuropein hexoside (18) all decreased in response to MeJA, while concentrations of unknown 1 (24) and unknown 2 (25) increased. Only fraxidin A (7) was affected in both experiments, although its concentration increased in 2008 and decreased in 2009 (Tables 2 and S1).

In white ash, concentrations of elenolic acid derivative 1 (3), oleuropein related compound 1 (12), and oleuropein A (16) increased following MeJA treatment in 2008, while verbascoside derivative 1 (21) and unknown phenylethanoid 1 (20) decreased (Tables 2 and S1). In 2009, tyrosol hexoside pentoside (4) and syringin (6) increased in concentration in response to MeJA, while concentrations of verbascoside derivative 1 (21) and kaempherol glucoside (23) decreased. The only compound in white ash that responsed to MeJA in a consistent manner in both years was verbascoside derivative 1 (21) (Tables 2 and S1).

EAB colonization of treated ash species in the common garden (Experiment 2)

Number of exit holes was affected by species and treatment with no significant species × treatment interaction (Fig. 1). Untreated white and green ash trees had more emergence holes than MeJA- and insecticide-treated trees (Fig. 1). Manchurian ash was very lightly colonized, as no tree had more than one EAB exit hole, regardless of treatment. Untreated black ash trees had 3.35 and 9.63 times more exit holes than MeJA- and insecticide-treated trees, respectively (Fig. 1).

Effect of MeJA treatment on individual phenolics, lignin, defense-related enzymes and rate of browning of trees in the common garden (Experiment 2)

Soluble phenolics were initially screened by analyzing each species individually across treatments, while lignin content was analyzed across both species and treatments from ash tree bark tissues harvested on 29 July 2009 from field grown trees.

Table 1 Phenolic compounds responding to induction with MeJA in at least one of three separate studies

Peak no.	Species and experiment[a]	RT-HPLC-UV[b]	λ max (nm)	$[M–H]^-$ or $[M–H]^+$ [c]	Fragments *m/z* (in order of decreasing abundance)[d]	Putative ID[e]	Reference[f]
1	M (1)	6.28	276.5	389	329, 167, 161	Vanillic acid hexoside acetate adduct A	Whitehill et al. (2012)
2	W (1,2)	7.34	275.3	299	179, 119, 143	Tyrosol hexoside	Eyles et al. (2007)
3	W (1)	8.76	267.7	601	403, 223	Elenolic acid derivative 1	Eyles et al. (2007)
4	W (2)	10.41	263.5, sh[g] 300	431	299, 191, 233	Tyrosol hexoside pentoside	Kammerer et al. (2005)
5	M (1)	10.89	290.7, 338.3	177	133	Esculetin C	Eyles et al. (2007)
6	W (2)	11.85	264.7	395*	233, 185	**Syringin**	**Standard match**
7	M (1,2)	12.58	290.7, 337.1	223*	208, 163, 107	Fraxidin A	Yasuda et al. (2006)
8	M (1)	13.10	sh 300, 341.9	369	207	**Fraxin**	**Standard match**
9	M (1)	14.14	291.9, 337.1	223*	208, 163, 107	Fraxidin B	Yasuda et al. (2006)
10	M (1)	15.46	328.7	385*	223	Mandshurin	Eyles et al. (2007)
11	M (1)	16.64	337.1	209*	149, 163, 181, 194	**Fraxetin**	**Standard match**
12	W (1)	26.34	278.9	525	363, 301, 345	Oleuropein related compound 1	Whitehill et al. (2012)
13	M (1)	28.15	326.4, 288.3	477	315, 203, 179, 341, 397	Calceolarioside C	Whitehill et al. (2012)
14	M (2)	28.30	278.8	ND[h]	ND	Unknown secoiridoid 1	Whitehill et al. (2012)
15	M (1)	33.65	328.7, sh 290	477	315, 323, 179, 341	Calceolarioside A	Eyles et al. (2007)
16	W (1)	35.65	sh 278	539	377, 275, 291, 359	Oleuropein A	Whitehill et al. (2012)
17	W (1,2,3), GP (3), GS (3)	36.11	331.1, sh 290	623	461	**Verbascoside**	**Standard match**
18	M (2)	36.31	280	701	539	Oleuropein hexoside	Eyles et al. (2007)
19	M (1)	39.23	327.6, sh 285	477	315, 281, 251, 179, 221	Calceolarioside B	Eyles et al. (2007)
20	W (1)	40.12	331, sh 300	ND	ND	Unknown phenylethanoid 1	Whitehill et al. (2012)
21	W (1,2)	41.54	315.6, sh 285	607	461	Verbascoside derivative 1	Eyles et al. (2007)
22	GP (3)	41.96	328.7, sh 290	623	461	Verbascoside B	Whitehill et al. (2012)
23	W (2)	42.41	353.8, 257.6, 266	447	285	Kaempherol glucoside	Chen et al. (2011)
24	M (2)	55.37	274.1, 341.8	119*	ND	Unknown 1	N/A
25	M (2)	57.17	296.6, 311, 286, sh 273	289*	ND	Unknown 2	N/A

[a] Letters indicate species (*M* Manchurian, *W* white, *GP* green 'Patmore', and *GS* green seedling) in which a specific compound was detected and number(s) in parentheses indicate the experiment in which a compound was affected by MeJA treatment: *1* 2008 containerized nursery experiment (Experiment 1), *2* 2009 containerized nursery experiment (Experiment 1), and *3* common garden field experiment (Experiment 2)

[b] *RT-HPLC-UV* Average retention time for compound from experiments in which it was significantly affected by MeJA treatment. If affected in several experiments, RT is an average of each global retention time from experiments where it was affected

[c] The dominant molecular ion $[M–H]^-$ detected via LC–MS. Compounds were run in parallel with a PDA detector and full scan MS_n mode. 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 in the positive ion mode, the corresponding $[M–H]^+$ is denoted with an asterik

[d] Corresponding fragmentation of the dominant molecular ion

[e] Tentative compound identity assigned based on literature match. Compound names in bold text were unequivocally confirmed with an external standard and are considered positive identifications

[f] Corresponding reference for which a compound has been previously described. Compounds were identified by HPLC-UV and LC–MS-PDA in bark tissues of ash trees treated with a 1-M solution of MeJA with 0.1 % Tween 20 in water

[g] *sh* Shoulder

[h] *ND* Not detected

Based on previously mentioned criteria, the total number of phenolic compounds chosen for statistical analyses across all taxa were as follows: 23 in black ash; 23 in green seedling; 19 in green 'Patmore'; 32 in Manchurian ash; and 23 in white ash. There was no effect of treatment on any of the phenolic compounds analyzed in black and Manchurian ash.

Our initial screening of phenolics within species revealed that our treatments affected the concentrations of only two compounds [verbascoside (17) and verbascoside B (22)]. Concentrations of verbascoside (17) in bark of trees belonging to the section Melioides (green and white ash) differed significantly among treatment groups: green 'Patmore' ($F_{2,21} = 11.262$; $P < 0.0001$), green seedling ($F_{2,23} = 8.144$; $P = 0.002$), and white ash ($F_{2,22} = 4.016$; $P = 0.034$) (Wallander 2008). Verbascoside (17) was identified in the bark of white ash, green 'Patmore', green seedling, and Manchurian ash, but it was not detected in black ash. Verbascoside (17) concentration differed among species, with Manchurian ash having the lowest concentration and white ash the highest (Fig. 2a). Across species, MeJA-treated trees had the highest concentration of verbascoside (17) and insecticide-treated trees the lowest, while water controls were intermediate.

Similarly, the concentration of verbascoside B (22) was higher in MeJA-treated green 'Patmore' ash (2.4 ± 0.3 mg g^{-1} FW) than in insecticide-treated trees (1.5 ± 0.1 mg g^{-1} FW), while water controls were intermediate (1.9 ± 0.2 mg g^{-1} FW). Verbascoside B (17) was detected in a few individuals of white and green seedlings, but quantities were below detection limit criteria in some cases, or present in only a few individuals in each treatment. Therefore this compound was excluded from further statistical analyses in these taxa.

Lignin concentration differed among species and across treatments, with no species × treatment interaction observed (Fig. 3a). Across all species, MeJA-treated trees contained higher levels of lignin than insecticide-treated trees, while water controls had intermediate concentrations (Fig. 3a). Within species, lignin concentration did not differ between treatment groups for green 'Patmore' ($F_{2,19} = 0.392$; $P = 0.681$) or Manchurian ash ($F_{2,20} = 1.973$; $P = 0.168$). In white ash and green seedlings lignin concentration tended to increase following MeJA treatment ($F_{2,23} = 3.246$; $P = 0.059$, and $F_{2,22} = 3.473$; $P = 0.058$, respectively), exhibiting the same response as the overall treatment effect (Fig. 3a). There were no differences in lignin concentration among black ash treatment groups ($F_{2,22} = 0.245$; $P = 0.785$).

Across species, the treatments had no effect on total protein concentration ($F_{2,73} = 0.545$; $P = 0.582$), rate of the browning reaction ($F_{2,73} = 0.368$; $P = 0.693$), chitinase ($F_{2,76} = 0.780$; $P = 0.462$), POD ($F_{2,71} = 1.50$; $P = 0.23$), or PPO activity ($F_{2,71} = 1.31$; $P = 0.276$). However, MeJA-treated plants had higher TI activity than insecticide-treated plants, with water-treated plants intermediate (Fig. 3c). Across species, green seedlings had the highest trypsin inhibitor activities, white ash was the lowest and Manchurian, black and green 'Patmore' ash intermediate. Chitinase activity varied among species across treatments ($F_{4,76} = 8.98$; $P = 0.0001$), with white ash having the highest activity. Total protein concentration also varied among species ($F_{4,73} = 2.99$; $P = 0.023$), with the two green ash taxa having higher concentrations than the three other species. Ash species did not differ in POD ($F_{4,71} = 1.57$; $P = 0.190$) and PPO activity ($F_{4,74} = 0.991$; $P = 0.445$), or rate of the browning reaction ($F_{4,73} = 2.07$; $P = 0.093$). The interaction between species and treatment was not significant for browning reaction ($F_{8,73} = 1.92$; $P = 0.069$), chitinase activity ($F_{8,76} = 1.0$; $P = 0.443$), POD activity ($F_{8,71} = 1.09$; $P = 0.38$), PPO activity ($F_{8,74} = 0.693$ $P = 0.599$), total protein concentration ($F_{8,73} = 0.953$; $P = 0.472$), or trypsin inhibitor activity (Fig. 3c).

In vitro larval bioassays

Survival of EAB larvae tended to decrease as verbascoside concentration in the diet increased (Fig. 2b). There was no difference in average mass of surviving larvae feeding on diets containing 0 or 5 mg g^{-1} verbascoside. Only one larva survived when feeding on the higher concentration of 20.9 mg g^{-1} verbascoside, reaching a mass of only 0.1 mg. No larvae survived when feeding on diets containing 45 mg g^{-1} verbascoside (Fig. 2b). As the concentration of lignin increased in diets, survival of EAB larvae also increased, but mass of surviving larvae tended to decrease (Fig. 3b). Concentration of soybean trypsin inhibitor (STI) had no effect on larval survival, although no larvae exposed to the highest STI concentration survived. Larval mass did, however, decrease as STI concentration increased (Fig. 3d).

Discussion

Where it has established in eastern North American forests, EAB has decimated green, white, and black ash (Klooster et al. 2014). Previous studies have shown Manchurian ash, which is endemic to Asia and shares a coevolutionary history with EAB, to be more resistant than these species, and have focused on identifying constitutive mechanisms of resistance (Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2011, 2012). In this study, application of MeJA to the bark of susceptible green, white, and black ash decreased colonization by EAB to levels similar to those of trees protected by bifenthrin insecticide. Increased resistance to EAB in MeJA-treated trees was associated with induction of general defense traits including changes in phenolic chemistry and accumulation of lignin and trypsin inhibitors in field grown ash. Analogs of lignin and trypsin inhibitors reduced growth of EAB larvae in artificial diets. The phenolic compound verbascoside consistently increased in concentration in bark tissues of white and green ash species following MeJA treatment. Furthermore,

Table 2 Quantities of phenolic compounds found in the bark of both Manchurian and white ash treated with 1 M MeJA or water (control) in Experiment 1

Peak no.	2008				2009			
	White		Manchurian		White		Manchurian	
	Control	MeJA	Control	MeJA	Control	MeJA	Control	MeJA
1	ND[a]	ND	0.3 (0.1) b	0.6 (0.1) a	ND	ND	0.7 (0.1) a	1.1 (0.1) a
2	0.5 (0.1) a	0.2 (0.01) b	0.5 (0.1) a	0.4 (0.04) a	1.2 (0.01) a	0.3 (0.01) c	0.6 (0.01) b	0.6 (0.01) bc
3	1.2×10^5 (1.4×10^4)[b] a	7.8×10^4 (7.5×10^3)[b] b	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	1.9 (0.01) a	1.6 (0.01) b	ND	ND
5	ND	ND	0.07 (2.0×10^{-3}) b	0.09 (0.01) a	ND	ND	0.1 (0.01) a	0.1 (0.01) a
6	6.7 (1.0) a	5.6 (0.4) a	ND	ND	10.5 (0.07) a	7.7 (0.03) b	ND	ND
7	ND	ND	0.6 (0.02) b	1.0 ± 0.1 a	ND	ND	2.1 (0.03) a	1.4 (0.02) b
8	ND	ND	11.6 (0.7) b	16.2 (0.02) a	ND	ND	25.5 (1.0) a	20.7 (0.8) a
9	ND	ND	0.3 (0.02) b	0.5 (0.02) a	ND	ND	0.7 (0.04) a	0.5 (0.03) a
10	ND	ND	2.9 (0.4) b	3.7 (0.3) a	ND	ND	4.7 (0.2) a	3.8 (0.3) a
11	ND	ND	1.0 ± (0.2) b	1.9 (0.1) a	ND	ND	1.5 (0.1) a	1.7 ± 0.2 a
12	3.9 (0.1) a	2.0 (0.4) b	ND	ND	ND	ND	ND	ND
13	ND	ND	1.0 (0.1) b	1.6 (0.1) a	ND	ND	3.7 (0.3) a	2.5 (0.2) a
14	ND	ND	ND	ND	ND	ND	1.0 (0.01) a	0.6 (0.01) b
15	ND	ND	18.2 (2.1) b	26.0 (1.2) a	ND	ND	27.7 (1.7) a	35.6 (2.1) a
16	2.8 (0.1)	ND	ND	ND	ND	ND	ND	ND
17	15.8 (0.4) b	43.4 (4.5) a	2.1 (0.4) c	3.3 (0.4) c	13.2 (0.3) b	39.0 (0.4) a	2.7 (0.04) c	5.4 (0.1) c
18	ND	ND	ND	ND	ND	ND	1.7 (0.02) a	0.9 (0.01) b
19	ND	ND	6.5 (1.2) b	10.9 (1.0) a	ND	ND	25.3 (2.5) a	16.4 (1.8) a
20	0.04 (0.02) b	0.6 (0.1) a	ND	ND	ND	ND	ND	ND
21	0.4 (0.01) b	0.7 (0.01) a	ND	ND	0.3 (0.01) b	0.9 (0.01) a	ND	ND
22	ND	ND	ND	ND	ND	ND	ND	ND
23	ND	ND	ND	ND	0.1 (7.0×10^{-4}) b	0.2 (0.1) a	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	8.8×10^3 (2.6×10^3)[b] a
25	ND	ND	ND	ND	ND	ND	ND	4.1×10^4 (8.0×10^3)[b] a
Lignin								
N/A	12.5 (2.1) b	14.6 (0.9) a	12.7 (1.6) b	14.7 (1.8) a	12.9 (1.1) a	17.8 (1.3) a	16.9 (2.1) a	16.0 (2.0) a

Peak numbers correspond to qualitative information reported in Table 1. Contents are expressed in mg g^{-1} FW (±SEM; $N = 8$) unless otherwise indicated. Different lowercase letters indicate significantly different means between a species and within a year (letters are compared within year for each individual compound and never between years). Means were separated post hoc by the Kruskal–Wallis test statistic (2008) or the protected LSD test ($\alpha = 0.05$) (2009)

[a] *ND* compounds not detected in a particular year/species

[b] Contents expressed as peak areas

when incorporated in an artificial diet at concentrations comparable to in planta, verbascoside decreased survival and growth of EAB neonates in a dose-dependent fashion, causing complete mortality at the highest concentration, which provides direct mechanistic evidence for the role of verbascoside in induced resistance in otherwise EAB-susceptible ash species.

These results suggest that the jasmonate pathway is a regulator of phenylpropanoids and some defense-related proteins in ash bark, and that jasmonates can be used to induce specific compounds that confer resistance to EAB in otherwise susceptible ash species. These responses are not surprising given that MeJA-induced elicitation of defense responses has been previously characterized in many plant

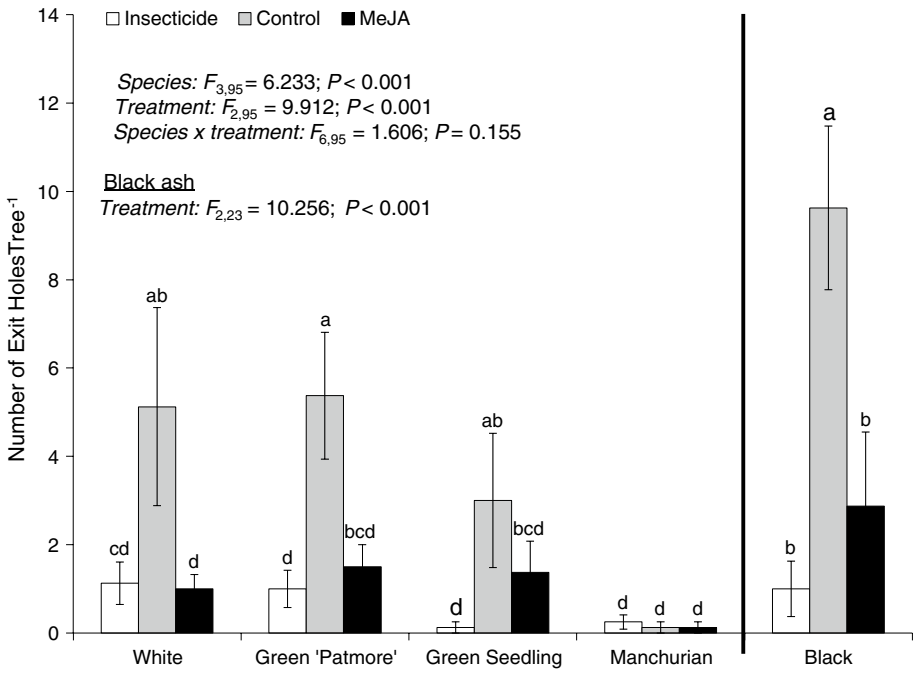

Fig. 1 Effects of species and treatment on EAB attack rates (mean number of emergence holes per tree ±SE) from Experiment 2. *Black ash* was not part of the original experimental design and was analyzed separately (*solid black line*). It is presented here for a visual comparison to the other species. *Different letters* indicate significantly different means separated by the protected LSD test ($\alpha = 0.05$)

species and includes the production of defense-related enzymes and specialized metabolites, such as phenylpropanoids (Farmer and Ryan 1990; Engelberth et al. 2004; Chen et al. 2005; Howe and Jander 2008). For example, a previous study demonstrated that MeJA could elicit changes in the volatile emission profiles of ash foliage following exogenous application of 0.3 % MeJA to Manchurian ash seedlings (15–30 cm tall) (Rodriguez-Saona et al. 2006).

Patterns of EAB emergence from resistant and susceptible species in our common garden study were consistent with the findings of Rebek et al. (2008), with untreated green, white, and black ash being significantly more susceptible than Manchurian ash. This pattern is also consistent with patterns observed in forests of southeast Michigan near the epicenter of the EAB invasion, where mortality of black, green, and white ash trees with stem diameters greater than 2.5 cm exceeded 99 % by 2009 (Klooster et al. 2014). Furthermore, when growing in the same vicinity in Asia, North American ash species suffer much higher mortality than Asian species (Wei et al. 2004). Black, green, and white ash may be particularly susceptible to EAB because they lack the targeted defenses of the coevolved host, Manchurian ash—the focus of several published investigations (Rebek et al. 2008; Whitehill et al. 2011, 2012; Herms and McCullough 2014).

Verbascoside accumulation was consistently induced by MeJA in the bark of white and green ash, in both containerized (Experiment 1) and field experiments (Experiment 2). In containerized nursery experiments, MeJA treatment increased the concentration of verbascoside in white ash by almost threefold in 2008 and 2009. The biosynthesis of verbascoside (acetoside) in olive (another member of the Oleaceae) occurs via tyrosol through hydroxytyrosol (Saimaru and Orihara 2010). Hydroxytyrosol hexoside was detected previously in constitutive ash bark samples (Whitehill et al. 2012). In containerized white ash trees, application of MeJA induced significant reductions of tyrosol hexoside, possibly because precursors were shunted toward the biosynthesis of verbascoside or other phenolics (Strack 1997). In the field experiment, MeJA application also increased concentration of verbascoside in white and green ash.

In our artificial diet studies, verbascoside almost completely inhibited survival of EAB larvae at medium to high concentrations, which are comparable to those observed in induced plants—and tended to affect EAB larvae at low doses. Other studies have found that bioassays amended with crude plant extracts containing verbascoside as a major component caused high levels of mortality in *Drosophila melanogaster* and *Spodoptera frugiperda* (Munoz et al. 2013). The results presented here are the first to provide evidence that purified verbascoside can function as an insecticidal agent against EAB larvae.

Patterns of lignin concentration among species (Experiments 1 and 2) were similar to previous studies (Cipollini et al. 2011; Whitehill et al. 2012). While diets amended with powdered lignin may not be the best proxy for the defensive function of cell wall-bound lignin in planta, the delayed development of larvae on lignin-amended diets supports its role as an anti-nutritive defense (Wainhouse et al. 1990). Lignin can act as an indirect chemical defense (Borg-Karlson et al. 2006), or a dose-dependent physical

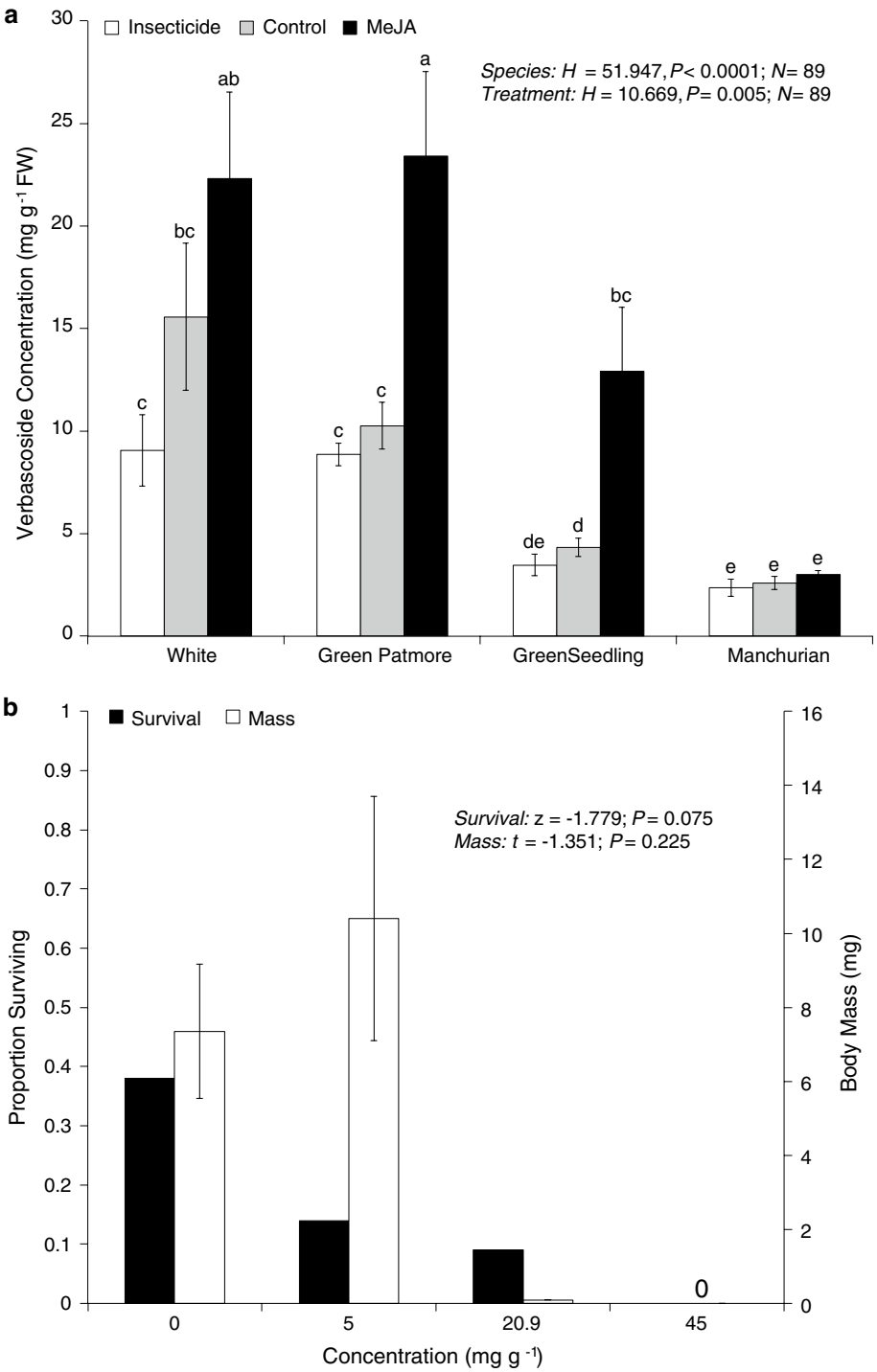

Fig. 2 Effect of MeJA on verbascoside and verbascoside on EAB larvae in vitro. **a** Concentration (mean ± SE) of verbascoside in bark tissues of white, green 'Patmore', green seedlings, and Manchurian ash from the common garden field experiment (Experiment 2). **b** Proportion surviving (*black bars*) and body mass (*white bars*) of EAB larvae reared on a bark-free artificial diet amended with varying concentrations of verbascoside—comparable to quantities observed in planta. *Different letters* indicate significantly different means separated by the protected LSD test ($\alpha = 0.05$). Bioassay data were analyzed using logistic regression

defense (i.e. sclereids) against wood-boring insects by reducing nutritional quality of tissues (Wainhouse et al. 1990; Franceschi et al. 2005; King et al. 2011). However, previous investigations found little evidence that interspecific variation in lignin content of bark tissues is associated with variation in constitutive resistance of ash to EAB (Cipollini et al. 2011; Whitehill et al. 2012). Our findings indicate that lignin may play a role in induced defense against EAB, yet further work is needed to identify the mechanism by which it exerts its effect.

Trypsin inhibitor (TI) activity was highest in MeJA-treated trees in the common garden experiment and may contribute to induced resistance against EAB. The defensive function of protease inhibitors such as TIs against insect herbivores is well documented (Green and Ryan 1972; Ryan 1990; Howe and Jander 2008), as is their

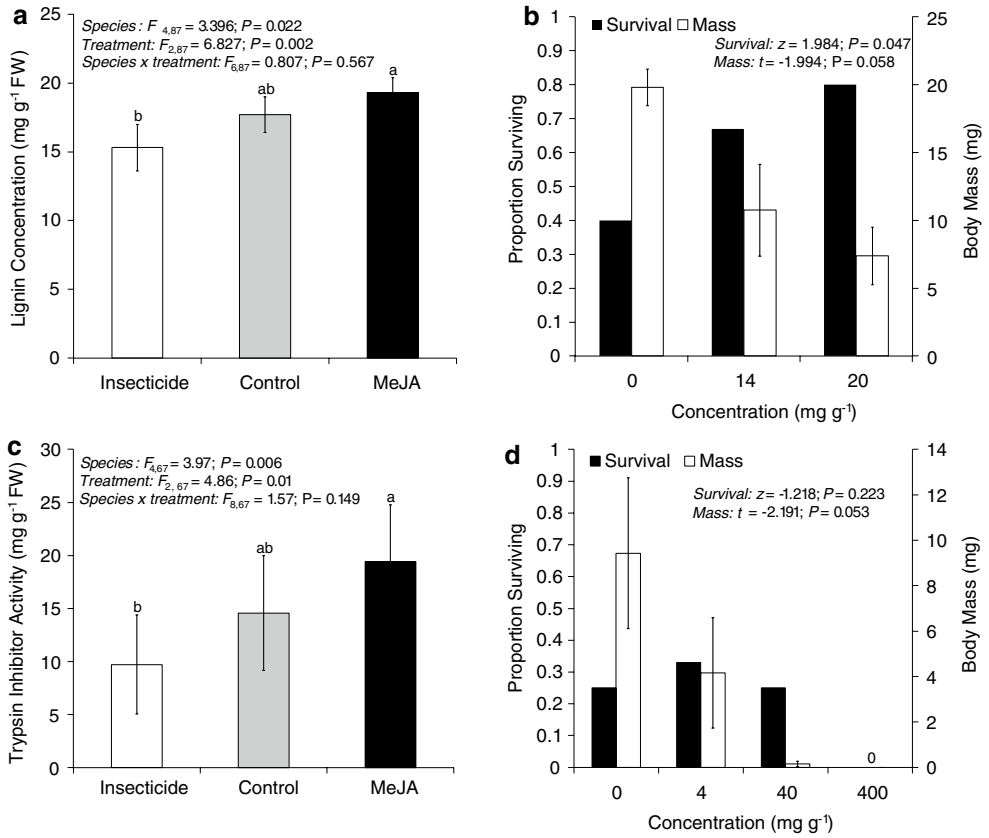

Fig. 3 Concentration of lignin and trypsin inhibitors in bark tissues of common garden field experiment ash (Experiment 2) and their effects on EAB larvae in vitro. **a** Total bark lignin concentration of ash species grown in the common garden, pooled by treatment. **b** Proportion surviving (*black bars*) and body mass (*white bars*) of EAB larvae reared on a bark-free artificial diet amended with different concentrations of lignin, comparable to those observed in planta. **c** Trypsin inhibitor activity in bark tissues of ash species grown in a common garden, pooled by treatment. **d** Proportion surviving (*black bars*) and body mass (*white bars*) of EAB larvae reared on a bark-free artificial diet amended with different concentrations of trypsin inhibitor comparable to those observed in planta. *Error bars* standard error of the mean in all panels. *Different letters* indicate significantly different means separated by the protected LSD test ($\alpha = 0.05$). Bioassay data were analyzed using logistic regression

regulation by jasmonates (Farmer and Ryan 1992; Howe and Jander 2008). TIs reduce an insect's ability to assimilate protein by inhibiting gut proteases, which negatively affects their growth and development (Broadway and Duffey 1986; Cipollini et al. 2004; Zavala et al. 2004). Not surprisingly, larvae exposed to very high STI concentrations failed to survive after initially feeding on the diet. At lower concentrations, STI had no effect on survival, but larval mass decreased as STI concentration increased. While we do not know how similar STI is to the TI proteins found in ash species, transcripts of serine proteases have been found in the midgut of EAB and protease inhibitor transcripts have been found in *Fraxinus* species (Mittapalli et al. 2010; Bai et al. 2011). This suggests that compounds capable of inhibiting serine proteases may indeed be a significant component of the heightened resistance displayed by MeJA-treated trees. We did not observe any effect of MeJA application on activities of CHI, POD, PPO, rate of browning, or total protein content, and thus it seems unlikely that they were responsible for increased EAB resistance of MeJA-treated trees.

While results of the in planta experiments suggest that increased resistance of white and green ash to EAB following MeJA treatment may have been due, at least in part, to increased quantities of verbascoside in bark tissues, the role of verbascoside in black and Manchurian ash resistance is doubtful because bark concentrations were low in these species with little difference in their concentration regardless of treatment (0 or <5 mg g FW, respectively). Increased resistance of black ash trees treated with MeJA may have been partly attributable to induction of TIs, as their activity in MeJA-treated and insect attacked untreated control trees were higher than the insecticide-treated trees. The lack of any induced responses (verbascoside, lignin, and TI) of Manchurian ash in the common garden to MeJA treatment suggests that mechanisms of resistance in Manchurian ash are likely constitutive (Whitehill et al. 2011, 2012), or unrelated to induction of phenylpropanoid metabolism and the defense proteins examined in this study.

Our study opens the way for further investigations of upstream regulators of the jasmonate pathway and enzymes involved in the biosynthesis of verbascoside in ash that could be used as biomarkers for traditional breeding or transgenic modification to enhance resistance. Our findings also provide the first direct evidence for a specific mechanism responsible for induced resistance of otherwise susceptible ash species to EAB larvae, which is the damaging life stage. Previous comparative studies that identified potential mechanisms responsible for the inherent resistance of Manchurian ash to EAB were only suggestive, as they identified interspecific variation in candidate constitutive resistance traits but did not confirm their role as defenses (e.g., Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2011, 2012).

Finally, MeJA could have the potential to be used as an insecticide alternative for control of EAB in ash belonging to the section Melioides. The use of MeJA to control insect pests is not a novel concept, but its application has been limited (Ryan 1990; Black et al. 2003). Further detailed experiments are required to confirm the potential of MeJA as an applied management tool of EAB in an urban or forest environment.

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