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Feeding by emerald ash borer larvae induces systemic changes in black ash foliar chemistry

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ABSTRACT

The exotic wood-boring pest, emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has been threatening North American ash (*Fraxinus* spp.) resources, this being recognized since its first detection in Michigan, USA and Ontario, Canada in 2002. Ash trees are killed by larval feeding in the cambial region, which results in disruption of photosynthate and nutrient translocation. In this study, changes in volatile and non-volatile foliar phytochemicals of potted 2-yr-old black ash, *Fraxinus nigra* Marshall, seedlings were observed in response to EAB larval feeding in the main stem. EAB larval feeding affected levels of six compounds [hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, methyl salicylate, and (*Z,E*)-α-farnesene] with patterns of interaction depending upon compounds of interest and time of observation. Increased methyl salicylate emission suggests similarity in responses induced by EAB larval feeding and other phloem-feeding herbivores. Overall, EAB larval feeding suppressed (*Z*)-3-hexenyl acetate emission, elevated (*E*)-β-ocimene emission in the first 30 days, but emissions leveled off thereafter, and generally increased the emission of (*Z,E*)-α-farnesene. Levels of carbohydrates and phenolics increased overall, while levels of proteins and most amino acids decreased in response to larval feeding. Twenty-three amino acids were consistently detected in the foliage of black ash. The three most abundant amino acids were aspartic acid, glutamic acid, glutamine, while the four least abundant were α-aminobutyric acid, β-aminoisobutyric acid, methionine, and sarcosine. Most (16) foliar free amino acids and 6 of the 9 detected essential amino acids decreased with EAB larval feeding. The ecological consequences of these dynamic phytochemical changes on herbivores harbored by ash trees and potential natural enemies of these herbivores are discussed.

1. Introduction

In response to biotic and abiotic stress, many plants undergo significant physiological and biochemical changes such as alteration of source-sink relations and induction of formation of defensive compounds (Karban and Baldwin, 1997; Gatehouse, 2002; Thompson and Goggin, 2006; Chen et al., 2009; Chen and Poland, 2009a,b). Increasing evidence has further shown that plants respond differently to chewing and phloem-feeding herbivores by triggering different signal transduction pathways (Walling, 2000). Most of these studies, however, focus on plant responses to foliage feeders (Karban and Baldwin, 1997; Orians et al., 2000; Rodriguez-Saona et al., 2009), and relatively few studies on plant responses to wood-borers in the trunk are available in the literature (but see Paine et al., 1997). It is well-known that locations of either wounded plant parts or vascular connectivity affect the magnitude of plant responses (Orians et al., 2000; Rodriguez-Saona et al., 2009).

One of the many common plant responses to herbivore wounding is increased emission of herbivore-induced volatiles and production of non-volatile compounds. Among those having induced levels are green leaf volatiles, monoterpenes, and sesquiterpenes (Paré and Tumlinson, 1997; Chen et al., 2008; Rodriguez-Saona et al., 2009). Many non-volatile defensive compounds are also induced by herbivory, both locally and systemically, e.g. terpenoid aldehydes in *Gossypium hirsutum* (Chen et al., 2008), sesquiterpenes in *Pinus nigra* (Wallis et al., 2008), polyphenols in *Quercus robur* (Scalbert and Haslam, 1987), polyphenol oxidase in *Solanum tuberosum* (Thipyapong et al., 1995), and pyrrolizidine alkaloids in *Senecio jacobaea* L. (Asteraceae) (Hol et al., 2003). Damage by many wood-boring bark beetles (Scolytidae) also significantly changes volatile and non-volatile chemical profiles of many pine trees (Byer, 1989).

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The exotic wood-boring EAB, probably originating from Asia through wood packing material, has been threatening the North American ash (*Fraxinus* spp.) resource since its first detection in Michigan, USA, and Ontario, Canada, in 2002 (Poland and McCullough, 2006). Phenolics have been suggested as possible agents of ash resistance to EAB (Eyles et al., 2007), and levels of phenolics in green ash, *F. pennsylvanica* Marsh, were induced by biotic and abiotic stress (Chen and Poland, 2009b). EAB larval feeding resembles girdling (removal of a ring of bark and phloem around the outer circumference of a tree) in many ways. For instance, both practices wound the phloem of a tree and interrupt the upward transport of nutrients and downward transport of photosynthates. Both practices cause crown dieback and epicormic shoots and eventually kill the tree in 3–5 years. Girdling is known to cause accumulation of carbohydrates and reduction in levels of amino acids or proteins above the girdled site (Noel, 1970; Roper and Williams, 1989; Li et al., 2003). Therefore, we have hypothesized that EAB larval feeding would: (1) increase black ash (*F. nigra* Marshall) foliage volatile emission and production of soluble phenolics, and (2) increase levels of foliar carbohydrates but decrease amounts of foliar proteins and amino acids.

2. Results

2.1. EAB larval feeding effects on headspace leaf volatile profiles

Among the 12 consistently detected volatile compounds, (*Z*)-3-hexenyl acetate (**3**) and (*Z,E*)-α-farnesene (**12**) were the most abundant (Fig. 1 and Table 1). EAB larval feeding in stems of black ash seedlings interacted with observation time in production of 6 compounds [i.e., hexanal (**1**), (*E*)-2-hexenal (**2**), (*Z*)-3-hexenyl acetate (**3**), (*E*)-β-ocimene (**4**), methyl salicylate (**5**), and (*Z,E*)-α-farnesene (**12**)] with patterns of interaction depending upon compounds of interest (Fig. 1 and Table 1). Interestingly, EAB larval feeding suppressed (*Z*)-3-hexenyl acetate (**3**) emission overall. EAB larval feeding within 30 days elevated (*E*)-β-ocimene (**4**) emission while its emission leveled off thereafter. The emission of methyl salicylate (**7**) and (*Z,E*)-α-farnesene (**12**) was generally increased by EAB larval feeding. Neither EAB larval feeding nor observation time affected emission of nonanal (**5**), benzyl cyanide (**6**), indole (**8**), β-caryophyllene (**9**), α-humulene (**10**), or α-cubenene (**11**) (Table 1).

2.2. EAB larval feeding effects on total proteins and carbohydrates

The total amount of proteins in leaves from control seedlings increased over time (Fig. 2). EAB larval feeding significantly decreased the total amount of proteins over the entire sampling period, and although it also increased over time, it did not increase as much as in control seedlings (Fig. 2). The levels of both glucose and starch were elevated by EAB larval feeding but the effect on starch levels was much greater (Fig. 3A and B). As a result of these increases, the level of total non-structural carbohydrates (sum of glucose and starch) was elevated in seedlings with EAB larval feeding (Fig. 3C).

2.3. EAB larval feeding effects on soluble phenolics

HPLC-UV chromatographic profiles of control and EAB-damaged black ash foliar soluble phenolics are shown in Fig. 4 (with structures in Fig. 5). Identification and characterization of phenolic compounds is presented in Table 2. Full spectra of identified phenolics are presented in S17–S28. Among the 12 identified phenolic compounds, three [i.e., esculin (**16**), verbascoside (**20**), and ligustroside (**22**)] were consistently detected (Table 2). Levels of verbascoside (**20**) was significantly induced after 20 days of EAB larval feeding, whereas amounts of other compounds were induced by EAB larval feeding at various observation times (Table 3).

2.4. EAB larval feeding effects on amino acids

Twenty-three amino acids were consistently detected in the foliage of black ash. They were alanine (**25**), α-aminobutyric acid (**26**), β-aminoisobutyric acid (**27**), asparagine (**28**), aspartic acid (**29**), glutamic acid (**30**), glutamine (**31**), glycine (**32**), glycine-proline (**33**), histidine (**34**), allo-isoleucine (**35**), leucine (**36**), lysine (**37**), methionine (**38**), ornithine (**39**), phenylalanine (**40**), proline (**41**), sarcosine (**42**), serine (**43**), threonine (**44**), tryptophan (**45**), tyrosine (**46**), and valine (**47**). The 3 most abundant amino acids of black ash were aspartic acid (**29**), glutamic acid (**30**), glutamine (**31**) (Fig. 6), and the least abundant were α-aminobutyric acid (**26**), β-aminoisobutyric acid (**27**), methionine (**38**), and sarcosine (**42**) (Fig. 7). EAB larval feeding in the trunk significantly decreased the concentrations of 16 foliar amino acids (all except β-aminoisobutyric acid (**27**), glycine-proline (**33**), histidine (**34**), ornithine

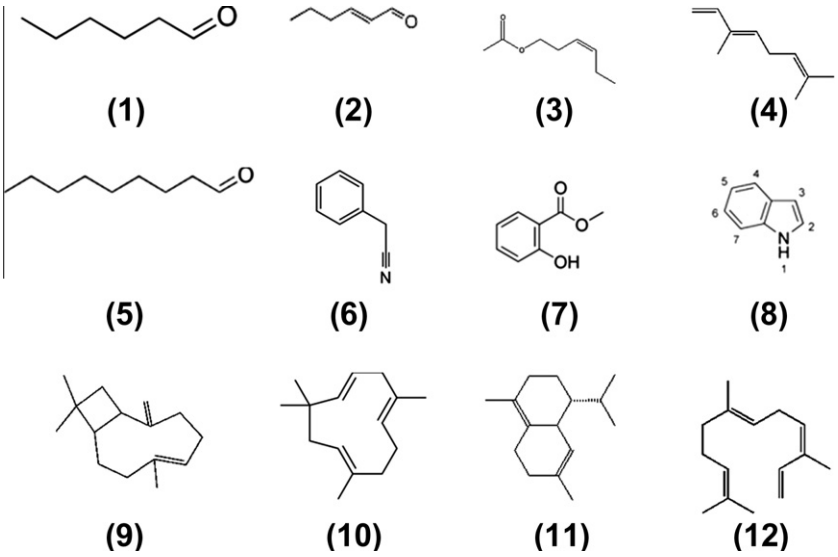

Fig. 1. Chemical structures of black ash (*Fraxinus nigra*) foliar volatile compounds. (**1**) hexanal; (**2**) (*E*)-2-hexenal; (**3**) (*Z*)-3-hexenyl acetate; (**4**) (*E*)-β-ocimene; (**5**) nonanal; (**6**) benzyl cyanide; (**7**) methyl salicylate; (**8**) indole; (**9**) β-caryophyllene; (**10**) α-humulene; (**11**) α-cubenene; (**12**) (*Z,E*)-α-farnesene.

Table 1
Black ash head-space foliar volatile compounds (mean ± 1SE ng g^{-1} dry tissue) in response to EAB larval feeding.

Volatilecompound	10 DAH[a]		20 DAH		30 DAH		40 DAH		50 DAH		60 DAH	
	Control[b]	EAB	Control	EAB	Control	EAB	Control	EAB	Control	EAB	Control	EAB
Hexanal **1**	1.7 ± 0.5	3.0 ± 2.6	0.2 ± 0.1	0.9 ± 0.3*	0.7 ± 0.4	0.7 ± 0.5	0.1 ± 0.0*	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.2*
(*E*)-2-hexenal **2**	5.4 ± 1.7a	16.5 ± 12.0	2.2 ± 0.6ab	6.2 ± 1.4*	0.7 ± 0.3b	1.4 ± 0.4	1.6 ± 0.3ab	0.9 ± 0.2	11.3 ± 5.9a	1.9 ± 0.4	2.6 ± 0.6ab	15.6 ± 6.7
(*Z*)-3-hexenyl acetate **3**	13.8 ± 9.2b	11.8 ± 4.1	50.1 ± 11.4a*	4.6 ± 1.4	15.9 ± 4.2b	8.3 ± 3.2	127.2 ± 48.6a*	19.8 ± 4.5	35.6 ± 13.3b*	0.9 ± 0.3	79.6 ± 25.3a*	8.0 ± 2.8
(*E*)-*β*-ocimene **4**	0.8 ± 0.3	17.3 ± 7.6*	3.7 ± 1.7	32.2 ± 8.2*	2.2 ± 0.7	90.7 ± 46.6*	3.6 ± 2.7	2.6 ± 0.7	1.8 ± 0.9	0.7 ± 0.3	3.3 ± 1.0	4.0 ± 0.7
Nonanal **5**	2.1 ± 1.1	4.4 ± 2.4	0.8 ± 0.2	8.3 ± 7.2	0.5 ± 0.2	0.8 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.5 ± 0.3
Benzyl cyanide **6**	2.2 ± 1.5	1.1 ± 0.8	0.0 ± 0.0	11.8 ± 11.8	0.1 ± 0.0	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.2
Methyl salicylate **7**	3.8 ± 3.0	4.7 ± 1.7	8.0 ± 2.5	20.7 ± 4.8*	1.9 ± 0.8	31.1 ± 8.1*	5.0 ± 2.4	16.7 ± 5.0	5.3 ± 1.5	2.4 ± 1.1	4.0 ± 0.7	27.5 ± 22.6
Indole **8**	0.2 ± 0.1	0.8 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
β-caryophyllene **9**	2.8 ± 1.2	7.9 ± 6.7	9.0 ± 3.5	7.4 ± 4.0	5.7 ± 2.5	32.7 ± 20.5	10.0 ± 3.6	2.2 ± 0.7	1.7 ± 0.7	0.1 ± 0.0	9.5 ± 5.2	0.6 ± 0.2
α-humulene **10**	0.4 ± 0.2	1.2 ± 0.7	1.7 ± 0.8	1.3 ± 0.5	0.9 ± 0.4	0.3 ± 0.1	1.2 ± 0.6	0.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	1.0 ± 0.7	0.3 ± 0.1
α-cubenene **11**	1.1 ± 0.4	1.8 ± 0.6	4.6 ± 3.0	6.4 ± 1.6	3.1 ± 1.8	24.3 ± 15.4	3.2 ± 1.2	3.2 ± 0.5	1.7 ± 0.7	0.5 ± 0.2	1.2 ± 0.2	8.4 ± 5.2
(*Z*,*E*)-*α*-farnesene **12**	72.9 ± 22.7	146.6 ± 48.1	131.0 ± 40.8	547.7 ± 89.7*	105.5 ± 53.2	1407.7 ± 769.6	58.2 ± 9.6	156.9 ± 24.5*	39.2 ± 15.1	34.5 ± 17.5	37.7 ± 7.1	432.0 ± 193.4

[a] DAH: days after egg hatching on EAB-damaged seedlings.
[b] Different lower-case letters following means denote significant difference between observations of Control plants at $\alpha = 0.05$.
* Denote significant difference between control and EAB-damaged plants within the same observation $\alpha = 0.05$; 10 DAH: $n_{EAB} = 4$, $n_{Control} = 6$; 20 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 30 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 40 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 50 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 60 DAH: $n_{EAB} = 3$, $n_{Control} = 6$.

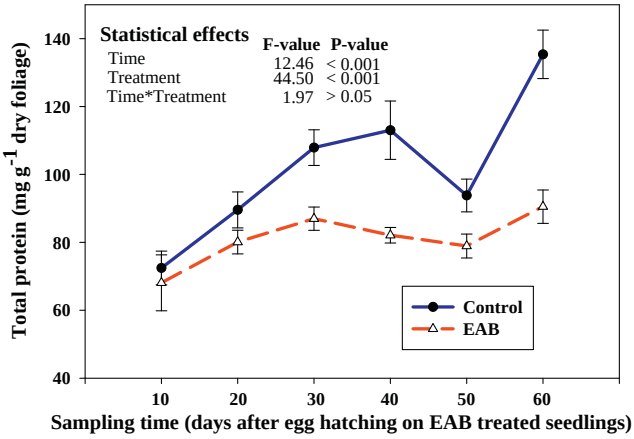

Fig. 2. Chemical structures of black ash (*Fraxinus nigra*) foliar volatile compounds. (**1**) hexanal; (**2**) (*E*)-2-hexenal; (**3**) (*Z*)-3-hexenyl acetate; (**4**) (*E*)-*β*-ocimene; (**5**) nonanal; (**6**) benzyl cyanide; (**7**) methyl salicylate; (**8**) indole; (**9**) *β*-caryophyllene; (**10**) *α*-humulene; (**11**) *α*-cubenene; (**12**) (*Z*,*E*)-*α*-farnesene.

(**39**), sarcosine (**42**), threonine (**44**), and tryptophan (**45**) among the 23) (Figs. 6 and 7, and S1–S16). The concentrations of most (17 out of 23) amino acids also changed over time. The patterns of change, however, differed depending on the particular amino acid. For example, the concentration of tryptophan (**45**) was high at day 10 and low at day 60, while that of lysine (**37**) was higher at day 30 and 40 than at any other time (S1–S16).

3. Discussion

Evidence has been accumulating that plants respond differently to chewing and phloem-feeding herbivores by triggering different signal transduction pathways, although cross-talk can occur between these pathways (van Wees et al., 2000; Walling, 2000). Activation of the octadecanoid pathway leading to the synthesis of jasmonic acid (JA) and its derivatives is a typical plant response to chewing herbivores, while the salicylic acid (SA) dependent signaling pathway is typically activated by biotrophic pathogen attack or pathogen-like damage caused by phloem-feeding insects (Walling, 2000). Emission of methyl salicylate (**7**), the volatile form of SA, was elevated by EAB larval feeding while methyl jasmonate was not detected, suggesting a possible similarity of host responses to EAB larval feeding and to phloem-feeding herbivores. However, to elucidate whether JA and/or SA are affected by EAB larval feeding, further studies focusing on these phytohormones are needed. EAB larval feeding, however, affected headspace foliar volatiles differently: overall, it suppressed (*Z*)-3-hexenyl acetate (**3**) emission; EAB larval feeding elevated (*E*)-*β*-ocimene (**4**) emission within 30 days but its emission level leveled off thereafter; and it generally elevated the emission of (*Z*,*E*)-*α*-farnesene (**12**). The effects of induction of (*E*)-*β*-ocimene (**4**) in the early stage of EAB larval feeding on black ash herbivores and natural enemies of these herbivores remain largely unknown, although antennal responses to this compound have been found for some herbivores such as sphinx moth *Manduca sexta* (Lepidoptera: Noctuidae) (Fraser et al., 2003) and EAB (Rodriguez-Saona et al., 2006). Similarly, the ecological function of (*Z*)-3-hexenyl acetate (**3**) is unknown.

The differential effect of EAB larval feeding in the trunk of black ash on foliar volatiles, compared to significant elevation of many foliar volatiles in response to EAB adult feeding on Manchurian ash foliage (Rodriguez-Saona et al., 2006), and white ash (*F. americana* L.) foliage (Chen et al., 2011b) suggests that ash species might respond to herbivory in the trunk and on the foliage differently.

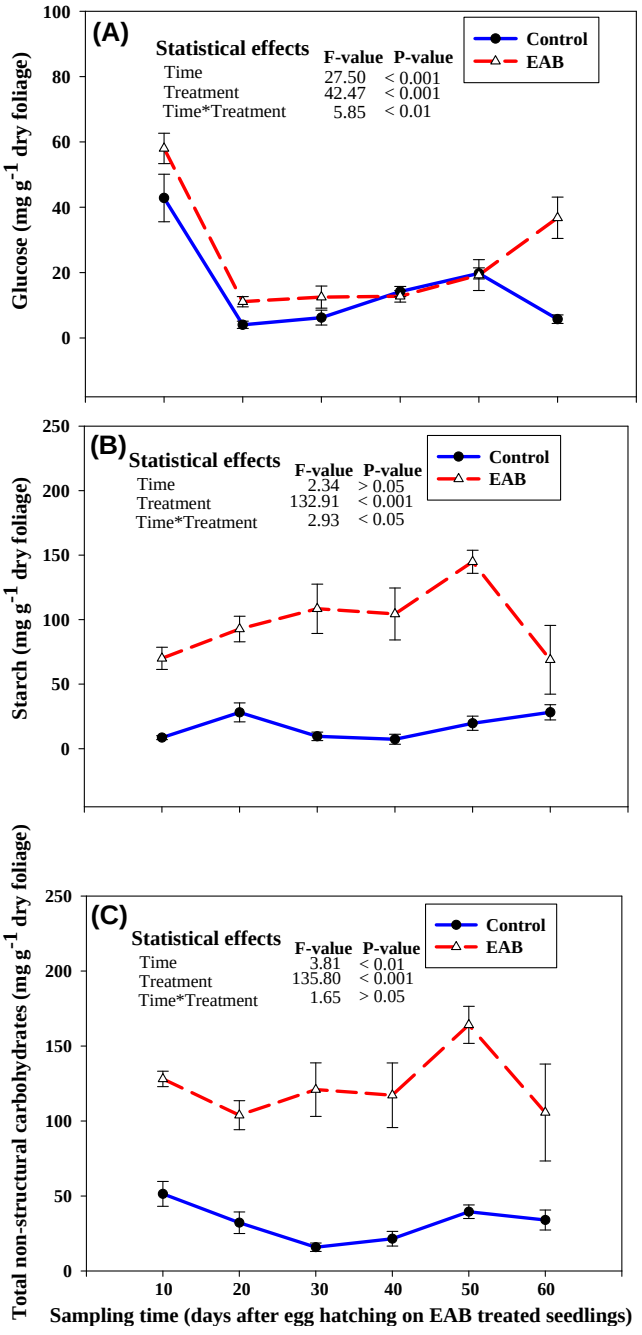

Fig. 3. Black ash foliar non-structural carbohydrates (sum of glucose and starch), glucose, and starch (mean ± SE mg g^{-1} dry foliage) in response to EAB larval feeding. Treatments: control (solid blue line) and EAB-damaged (dashed red line); (A) glucose; (B) starch; (C) total non-structural carbohydrates; 10 days after hatching of EAB-treated seedlings (DAH): $n_{EAB} = 4$, $n_{Control} = 6$; 20 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 30 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 40 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 50 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 60 DAH: $n_{EAB} = 3$, $n_{Control} = 6$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Species-specificity in response to herbivory is also likely an important factor.

We found an increase in foliar carbohydrates (Fig. 3) and a decrease in total proteins (Fig. 2) and most amino acids (Figs. 6 and 7, and S1–S16), in response to EAB larval feeding in the trunk of black ash. In many cases, amino acid levels in plants and animals are elevated by stress (Good and Zaplachinski, 1994; Bischof, 1996; Dadmarz et al., 1998). In our study, the decrease in proteins and amino acids was probably due to a combination of reduced synthesis and increased export of these compounds from foliage to a new nutrient sink below the EAB larval feeding site. This suggestion is supported by evidence from another study investigating how EAB larval feeding affects nutritional qualities of phloem below and above feeding sites in the same ash species (Chen et al., 2011a). In that study, we found EAB larval feeding decreased most amino acids both below and above feeding sites. However, the levels of most amino acids were significantly higher in the phloem below the feeding site compared to above. A typical symptom of EAB infestation is the outgrowth of epicormic shoots below the EAB larval feeding location (Poland and McCullough, 2006). Epicormic shoots, or water sprouts, arise from dormant or adventitious buds embedded in the bark of woody plant trunks. Their development is normally inhibited by the plant hormone auxin flowing downward from crown foliage under normal conditions (Wignall and Browning, 1988; Creber and Collinson, 2006). Many biotic and abiotic factors such as loss of foliage trigger the release of the plant hormone indole-3-acetic acid (IAA) in the cambial region, which subsequently stimulates the development of these buds into epicormic shoots (Wignall and Browning, 1988; Burrows, 1989; Creber and Collinson, 2006; Gordon et al., 2006). Higher levels of amino acids below the feeding site than above the feeding site may be due to export of nutrients to support the rapid growth of these epicormic shoots. However, the modulator of these responses, either EAB larvae or the host ash seedlings, remains unknown since these responses appear to benefit both sides.

We profiled black ash foliar soluble phenolics and their response to EAB larval feeding in the trunk. Compared to ash phloem tissues of previously studied ash species (green, Manchurian, and white ash; Eyles et al., 2007), black ash foliage appeared to contain fewer phenolic compounds. Nevertheless, as predicted, levels of many phenolics were also elevated by EAB larval feeding (Table 3).

Systemic changes in foliar chemistry induced by EAB larval feeding in the trunk could have profound effects on the interactions of black ash with EAB and other herbivores of black ash. Nitrogen (in the form of protein) is a limiting factor for plant and animal growth in nature (White, 1993). A decrease in the ratio of proteins to carbohydrates (which is considered an index of nutrient balance – Waldbauer and Friedman, 1991; Behmer, 2009) may make black ash foliage less nutritious and less palatable to foliage feeders. Many herbivores are known to choose more nutritionally-balanced food than less nutritionally-balanced food in feeding preference trials on artificial diets (Waldbauer and Friedman, 1991; Behmer, 2009). We also hypothesize that the palatability of black ash foliage is further deteriorated by an elevation of phenolics in trees damaged by EAB larval feeding (Table 3). Therefore, colonization of black ash trees by foliage-feeders including EAB adults might be deterred, or the development of these herbivores might be retarded, by a combination of increased defensive compounds and lower nutritional balance. Colonization or development of sap-feeding insects such as mites might also be detrimentally affected because levels of most physiologically free amino acids, many of which are essential amino acids, of the foliage were also lowered by EAB larval feeding and sap-feeders utilize these amino acids almost exclusively.

4. Conclusions

Feeding by larvae of the invasive EAB in the trunk of black ash seedlings induced systemic phytochemical changes in ash foliage. This study provides base knowledge for subsequent studies on interactions between ash trees, EAB, other herbivores, and probably natural enemies of herbivores. The mechanisms of these systemic physiological and biochemical changes due to EAB larval

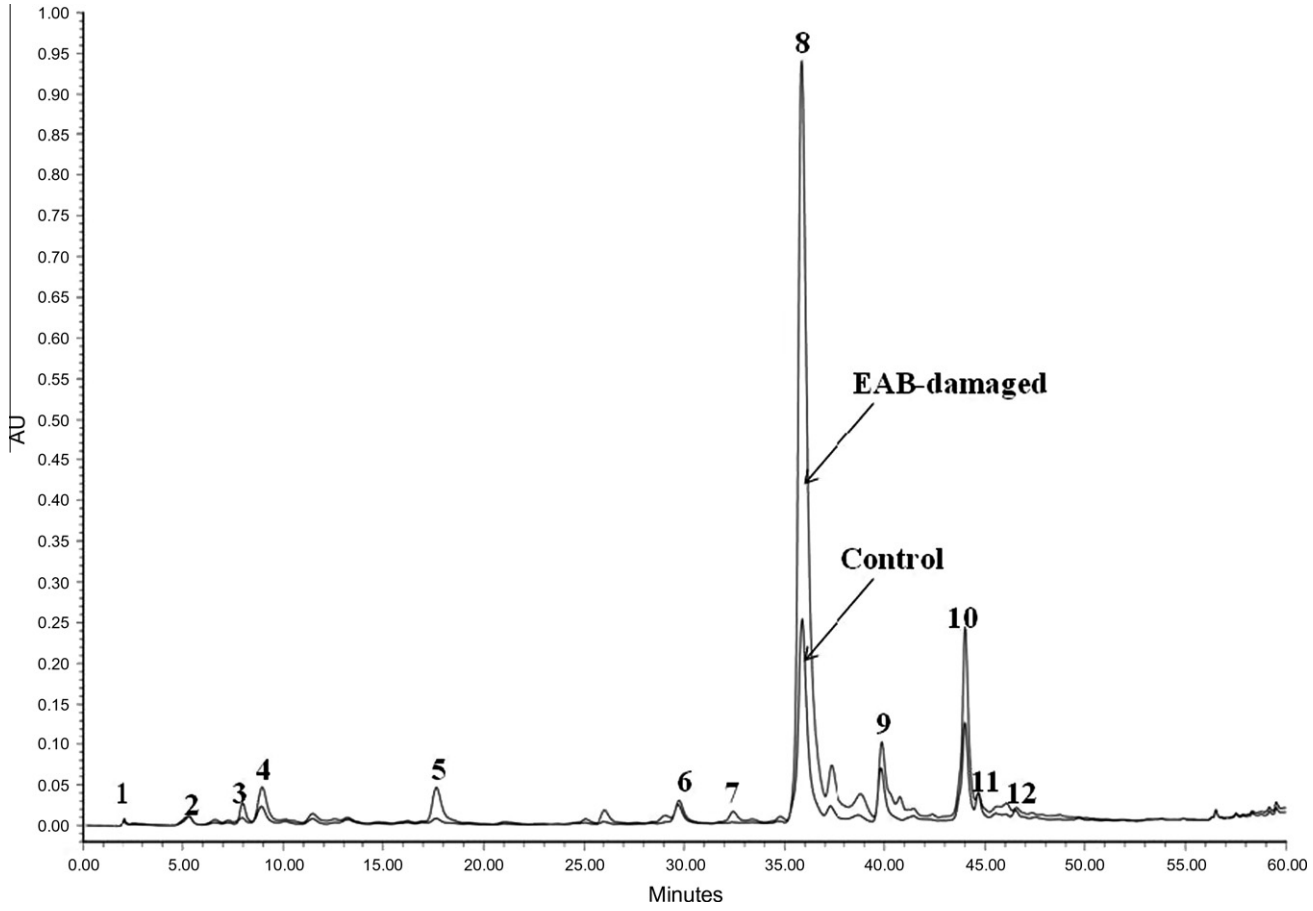

Fig. 4. HPLC-UV chromatographic profiles at 280 nm of methanol extracts of black ash foliage. Peak 1: homovanillic acid (**13**); 2: hydroxytyrosol hexoside (**14**); 3: tyrosol hexoside (**15**); 4: esculin (**16**); 5: caffeoyl-quinic acid (**17**); 6: 10-hydroxy-oleuropein (**18**); 7: pinoresinol (**19**); 8: verbascoside (**20**); 9: oleuropein (**21**); 10: ligstroside (**22**); 11: kaempherol galactoside (**23**); 12: kaempherol rhamnosylhexoside (**24**) (see Tables 2 and 3).

feeding remain unknown. The elicitors could be mechanical boring and chewing, or EAB salivary and frass components. Further studies are clearly needed to elucidate these mechanisms, since systemic phenomena are hypothesized to exert fundamental ecological effects in trees (Bonello et al., 2006).

5. Experimental

5.1. Ash seedlings and EAB eggs

All native ash species in the USA are susceptible to EAB. For the sake of easy manipulation, black ash seedlings were used in this study because the trunks were larger for pinning EAB eggs, compared to other species available. Age of black ash seedlings (*Fraxinus nigra*) and growing methods were described elsewhere (Chen et al., 2011a,b).

EAB eggs were prepared according to Chen et al., 2011a,b. Eggs together with a thin layer of ash bark were removed with a knife. Eggs used in each experiment were about 12- to 13-d-old.

5.2. Experimental setup

Seventy-two (72) ash seedlings, approximately 70 cm tall and 2 cm in trunk diameter at 30 cm above the growing soil, were selected and grown as described in Chen et al. (2011a). The seedlings were randomly assigned to either EAB or control treatments approximately 4 weeks after potting. EAB eggs were obtained by excising small pieces of bark with eggs attached from ash sticks on which females had oviposited in the laboratory. For treatment EAB, 6 eggs of the same age were attached to the main trunk of each seedling about 30 cm above the soil by gently pinning through the edge of the layer of attached ash bark using 4 #3 insect pins (ca. 0.05 cm in diameter). For control treatments, ash bark without eggs was pinned with the same number of insect pins. Six randomly selected seedlings from each treatment were sampled at 10-day-intervals beginning 10 days after the eggs had hatched and larvae had begun to feed on the EAB treatment (i.e., day 10, 20, 30, 40, 50, and 60). Because EAB eggs generally hatch in our lab within 17–18 days at 25 ± 2 °C after oviposition, and the eggs used in the experiments were 12- to 13-d-old, egg hatching did not occur until approximately 5 days after pinning. Hence, the first observations occurred 15 days after the eggs were pinned, corresponding to 10 days after egg hatching and initiation of feeding. Eggs were allowed to hatch and incur larval feeding damage because ash trees are killed by larval feeding in the cambial region, which results in disruption of photosynthate and nutrient translocation.

At the end of each observation, after volatile collection described below, the trunk bark of each seedling was peeled carefully to check the number of EAB larvae and their developmental stages. Each treatment (EAB or control) was originally replicated 6 times per observation time. For the EAB treatment, only those replicates in which at least one larva was observed were included in the EAB treatment data set, with an average of 1.7 larvae (the low EAB

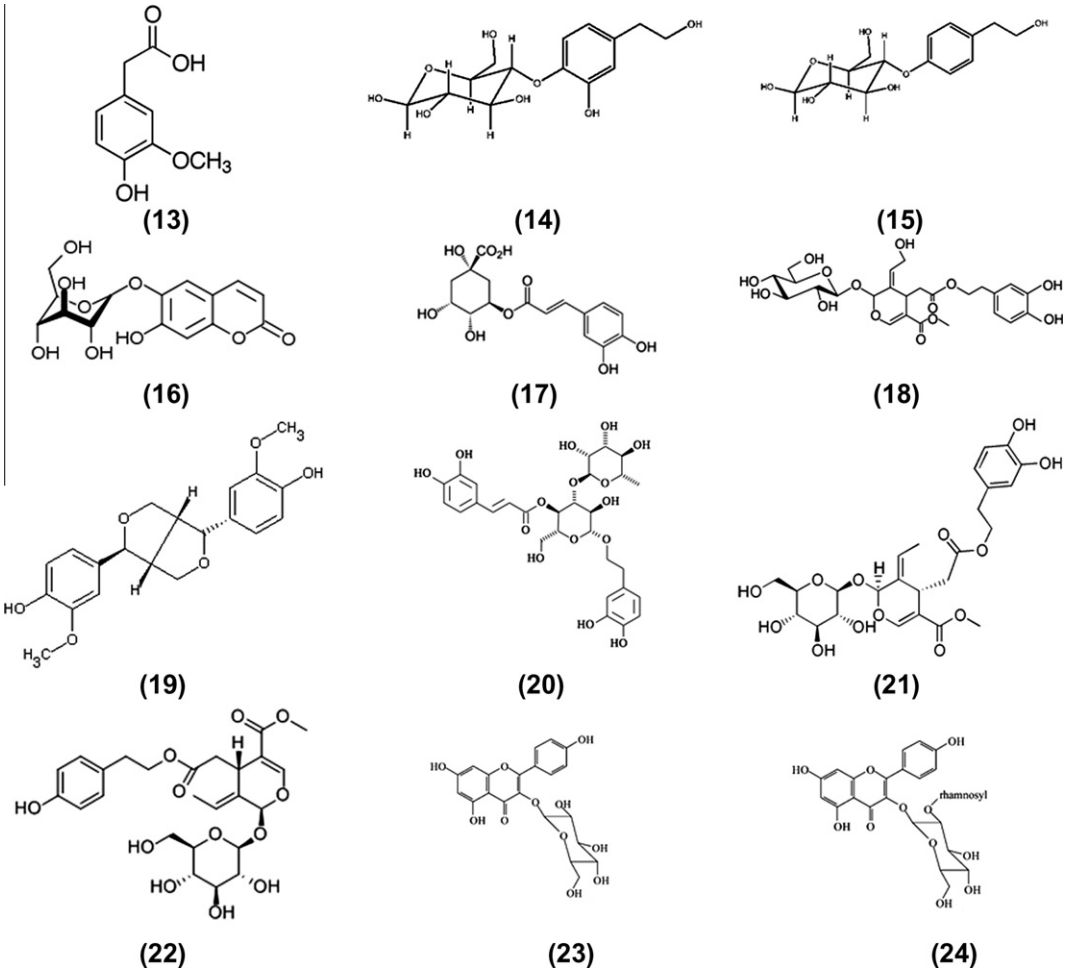

Fig. 5. Chemical structures of black ash (*Fraxinus nigra*) foliar soluble phenolics. (**13**): homovanillic acid; (**14**): hydroxytyrosol hexoside; (**15**): tyrosol hexoside; (**16**):esculin; (**17**): caffeoyl-quinic acid; (**18**): 10-hydroxy-oleuropein; (**19**): pinoresinol; (**20**): verbascoside; (**21**): oleuropein; (**22**): ligstroside; (**23**): kaempherol galactoside; (**24**): kaempherol rhamnosylhexoside.

Table 2
Identification of phenolic compounds from black ash based on retention time, *m/z* ratios, MS fragmentation, and UV absorbance maxima.

Peak #	Timepoint[a]	RT (min)	$[M–H]^-$ (*m/z*)	Fragments *m/z* (in order of decreasing abundance)	UV (λ) maxima(nm)	Compound ID	References
1	2	2.14	181	163, 101, 85	275.3	Homovanillic acid (**13**)	Ryan et al. (1999)
2	1, 6	5.108	315	153, 135, 123	280	Hydroxytyrosol hexoside (**14**)	Cardoso et al. (2005)
3	1, 4, 5	7.938	299	179, 119, 143, 161	276.5	Tyrosol hexoside (**15**)	Kammerer et al. (2005)
4	1, 2, 3, 4, 5, 6	8.9	339	177, 133	326.3, sh[b] 295	Esculin (**16**)	Parejo et al. (2004)
5	4, 5, 6	17.635	353	191, 179, 135	326.3, sh 295	Caffeoyl-quinic acid (**17**)	Cardoso et al. (2005)
6	1, 2, 3, 5, 6	29.605	555	273, 393, 307, 137	281.2	10-hydroxy-oleuropein (**18**)	Cardoso et al. (2005)
7	4	32.435	357	151, 136	278.8	Pinoresinol (**19**)	Ye et al. (2005)
8	1, 2, 3, 4, 5, 6	35.74	623	461, 315, 297, 135	331.1, sh 290	Verbascoside (**20**)	Ryan et al. (1999)
9	1, 2, 3, 4, 5, 6	39.772	539	307, 377, 275, 291	281.2	Oleuropein (**21**)	Tanahashi et al. (1998)
10	1, 2, 3, 4, 5, 6	43.967	523	291, 361, 259, 139, 111	sh 280	Ligstroside (**22**)	Ryan et al. (2002)
11	2, 5, 6	46.013	447	285, 175, 199, 241	350.2, sh 290, 265.8	Kaempherol galactoside (**23**)	Ye et al. (2005)
12	1	47.538	593	285, 327, 255	358.4, sh 280, 265	Kaempherol rhamnosylhexoside (**24**)	Ye et al. (2005)

[a] Time point 1–6 denotes 10, 20, 30, 40, 50, and 60 days after hatching on EAB damaged seedling, respectively.
[b] Shoulder.

average is due to low hatching rate of laboratory collected EAB eggs) per seedling. The actual damage was visually estimated to range from one to five square centimeters. The final replicates for the treatments were as follows: Day 10: $n_{EAB} = 4$, $n_{Control} = 6$; Day 20: $n_{EAB} = 6$, $n_{Control} = 6$; Day 30: $n_{EAB} = 5$, $n_{Control} = 6$; Day 40: $n_{EAB} = 6$, $n_{Control} = 6$; Day 50: $n_{EAB} = 5$, $n_{Control} = 6$; Day 60: $n_{EAB} = 3$, $n_{Control} = 6$.

5.3. Headspace leaf volatile collection and analysis

This experiment was designed to investigate the effects of EAB larval feeding and the duration of larval feeding on ash foliage volatile emission. Volatile compounds from terminal branch (containing 5–8 compound leaves) of each seedling were collected following methods described in Rodriguez-Saona et al. (2006)

Table 3
Black ash foliar phenolics (mean ± SEM mg g^{-1} dry weight) in response to EAB larval feeding.

Phenolicspeak #	Compound	10 DAH[a]		20 DAH		30 DAH		40 DAH		50 DAH		60 DAH	
		Control	EAB	Control	EAB	Control	EAB	Control	EAB	Control	EAB	Control	EAB
1	(**13**)	ND[b]	ND	0.3 ± 0.0	3.9 ± 0.9[d]	ND	ND	ND	ND	ND	ND	ND	ND
2	(**14**)	0.5 ± 0.1	2.1 ± 0.8	ND	ND	ND	ND	ND	ND	ND	ND	0.9 ± 0.1	2.9 ± 1.8
3	(**15**)	1.1 ± 0.3	9.4 ± 4.2	ND	ND	ND	ND	0.9 ± 0.3	0.4 ± 0.1	1.5 ± 0.7	0.5 ± 0.1	ND	ND
4	(**16**)	1.3 ± 0.5	2.8 ± 1.0	0.8 ± 0.3[c]	4.3 ± 1.6	1.6 ± 0.4	4.1 ± 1.1	2.2 ± 1.2	4.3 ± 0.5	1.5 ± 0.3	5.5 ± 2.3	1.2 ± 0.6	3.9 ± 0.8
5	(**17**)	ND	ND	ND	ND	ND	ND	ND	ND	0.2 ± 0.1	0.4 ± 0.1	0.0 ± 0.0[c]	4.8 ± 3.1
6	(**18**)	1.4 ± 0.2	1.1 ± 0.5	4.3 ± 0.3	10.5 ± 2.2	2.8 ± 0.7	4.5 ± 1.0	ND	ND	1.8 ± 0.5	3.6 ± 0.6	3.9 ± 1.8	6.5 ± 1.6
7	(**19**)	ND	ND	ND	ND	ND	ND	0.1 ± 0.0[e]	2.1 ± 0.4	ND	ND	ND	ND
8	(**20**)	20.9 ± 3.1	30.6 ± 7.3	21.6 ± 2.6[e]	81.5 ± 9.5	22.5 ± 3.0[e]	76.2 ± 11.6	24.7 ± 3.5[e]	93.9 ± 8.8	23.7 ± 2.9[d]	72.1 ± 13.8	37.0 ± 6.2[d]	117.0 ± 23.6
9	(**21**)	4.6 ± 1.4	2.9 ± 1.3	ND	ND	8.9 ± 1.1	14.7 ± 2.3	14.0 ± 3.8[c]	27.3 ± 6.8	4.2 ± 1.5	12.0 ± 2.6	11.1 ± 2.2	20.8 ± 1.4
10	(**22**)	12.2 ± 2.4	8.7 ± 3.3	ND	ND	11.0 ± 0.9[d]	22.4 ± 2.9	ND	ND	7.8 ± 1.6[d]	16.9 ± 1.7	13.9 ± 1.7	21.8 ± 3.0
11	(**23**)	ND	ND	0.7 ± 0.1[e]	1.9 ± 0.2	ND	ND	ND	ND	0.7 ± 0.2[d]	2.4 ± 0.9	0.7 ± 0.0[c]	1.7 ± 0.5
12	(**24**)	0.8 ± 0.3	1.5 ± 0.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] DAH: days after egg hatching on EAB-damaged seedlings.
[b] Below detection limit.
[c,d,e] $p < 0.05$, 0.01, and 0.001 between EAB and control treatments within the same observation, respectively; 10 DAH: $n_{EAB} = 3$, $n_{Control} = 4$; 20 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 30 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 40 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 50 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 60 DAH: $n_{EAB} = 3$, $n_{Control} = 6$.

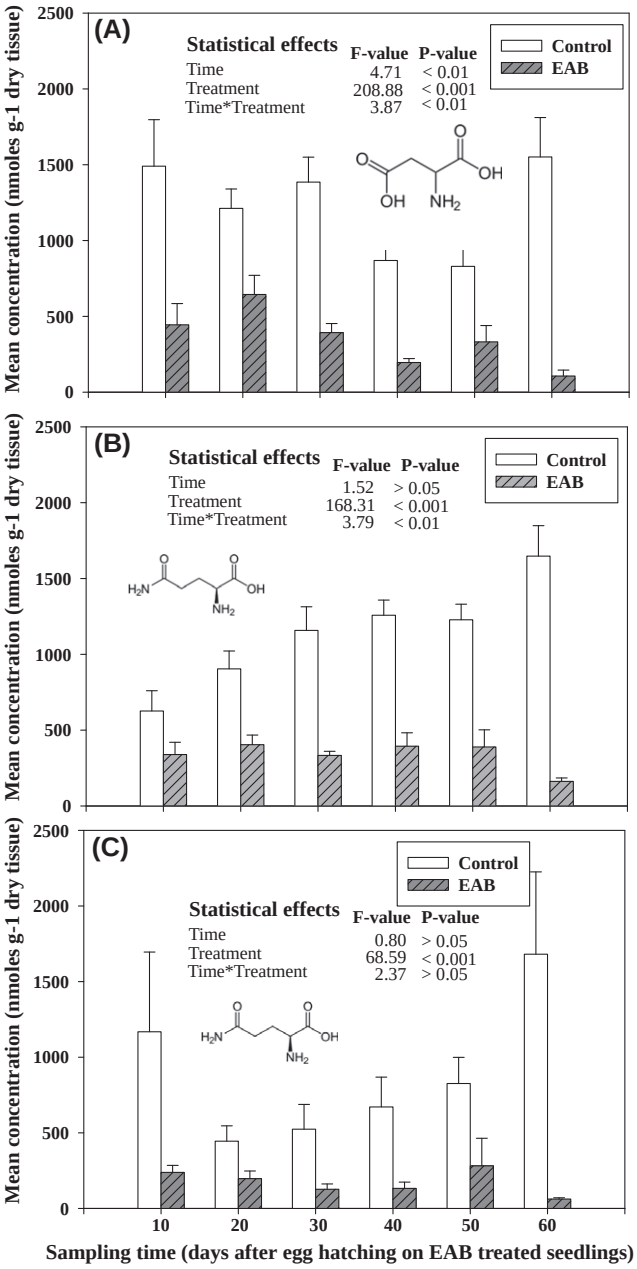

Fig. 6. Concentrations (mean ± SE n moles g^{-1} dry foliage) of the three most abundant black ash foliage amino acids in response to EAB larval feeding. Treatments: control (blank bars); EAB-damaged (shaded bars); (A) aspartic acid (**29**); (B) glutamic acid (**30**); (C) glutamine (**31**); 10 days after hatching of EAB-treated seedlings (DAH): $n_{EAB} = 4$, $n_{Control} = 6$; 20 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 30 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 40 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 50 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 60 DAH: $n_{EAB} = 3$, $n_{Control} = 6$.

and Chen et al. (2011b). Volatile compounds were extracted and analyzed according to methods described in Chen and Poland (2009a).

5.4. Leaf sample collection for non-volatile compound determination

Foliar non-volatile chemicals investigated were total proteins, carbohydrates (glucose and starch), total soluble phenolics, and amino acids. Immediately before volatile collection, a compound leaf that was not from the volatile collection terminal and had matured 1 wk before was collected from each seedling. The excision of one compound leaf had little effect on head space volatile

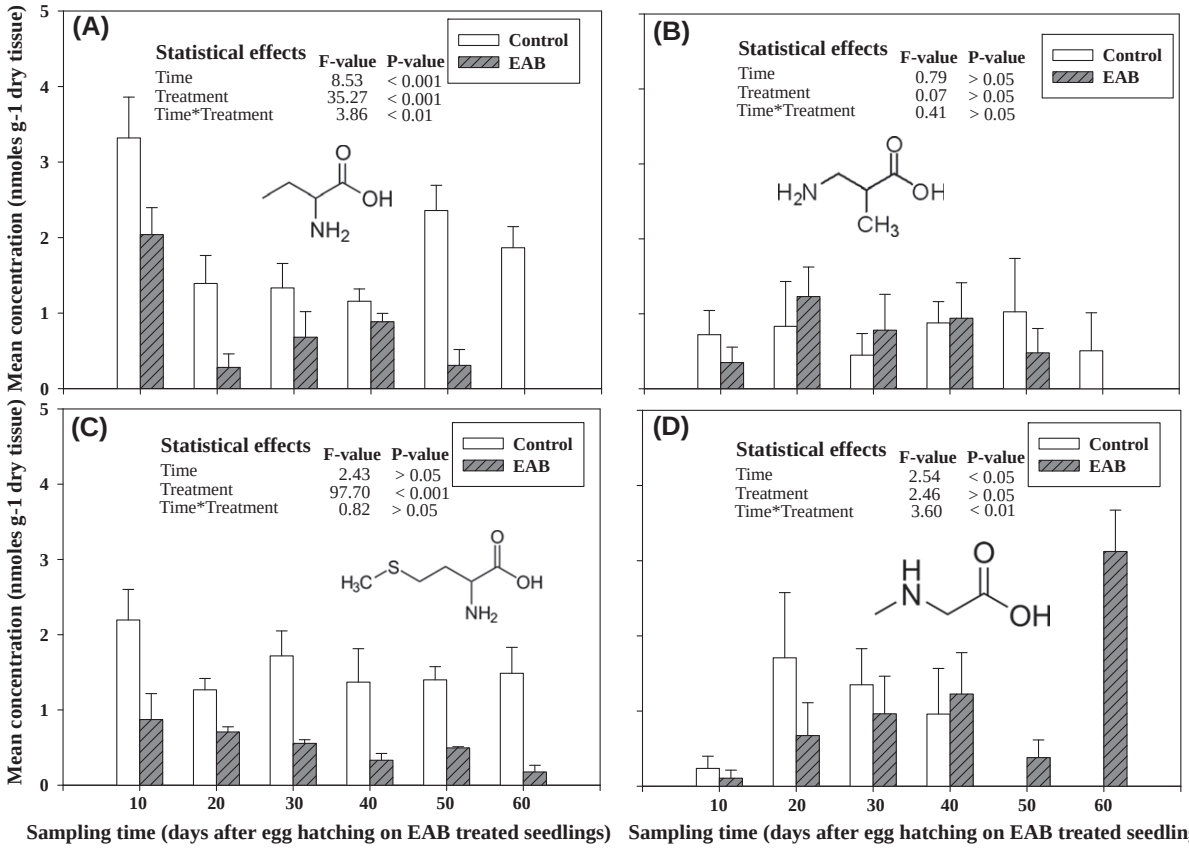

Fig. 7. Concentrations (mean ± SE n moles g^{-1} dry foliage) of the four least abundant black ash foliage amino acids in response to EAB larval feeding. Treatments: control (blank bars); EAB-damaged (shaded bars); (A) α-aminobutyric acid (**26**); (B) β-aminoisobutyric acid (**27**); (C) methionine (**38**); (D) sarcosine (**42**); 10 days after hatching of EAB-treated seedlings (DAH): $n_{EAB} = 4$, $n_{Control} = 6$; 20 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 30 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 40 DAH: $n_{EAB} = 6$, $n_{Control} = 6$; 50 DAH: $n_{EAB} = 5$, $n_{Control} = 6$; 60 DAH: $n_{EAB} = 3$, $n_{Control} = 6$.

emissions from black ash (unpublished data). Leaves were immediately dipped in liquid N_2 for 5 s and brought to the lab in a cooler containing dry ice. Samples were then stored in the freezer at −20 °C and lyophilized within 4 wks using a Modulyo® freeze dryer (Thermo Scientific, Pittsburgh, PA). Leaves were later ground to fine powder with a mortar and pestle.

5.5. Extraction and determination of total proteins and carbohydrates

Protein and carbohydrate extractions and determinations followed Chen and Poland (2009b).

5.6. Extraction and determination of foliar soluble phenolics

Foliar soluble phenolics extraction protocol followed Eyles et al., 2007, with analysis, identification of soluble phenolics, and quantification of identified compounds as for Chen et al. (2011b).

5.7. Extraction and analysis of foliar amino acids

Leaf samples were collected and prepared as in the preceding experiment. Amino acid extraction, derivatization, and analysis followed methods described by Chen et al. (2011a).

5.8. Statistical analysis

All data except phenolics were subjected to repeated measurement analyses because data collected over time were likely correlated (PROC MIXED in SAS; Littell et al., 2006). The statistical model is $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} denotes the measurement at time j on the k^{th} subject assigned to treatment i (i = EAB or control; j = 1 to 6; k = 1 to 6), and μ, α_i, β_j, $(\alpha\beta)_{ij}$, and ε_{ijk} denote overall mean, treatment effects, observation time effects, treatment and observation time interactions, and error term, respectively. The covariance structures were modeled using compound symmetry which assumes (1) equal variance at all observation times; and (2) equal covariance between subjects (seedlings) at all pairs of observation times (type = cs in REPEATED statement in SAS). The fit of the model was verified by fit statistics [i.e., −2 Res Log Likelihood, Akaike's information criterion (AIC), the AIC corrected (AICC), and Bayesian information criterion (BIC)]. Volatile data were square-root-transformed (Chen et al., 2008) and total proteins, glucose, starch, TNC, and amino acid data were natural-logarithm-transformed to meet the normality and equal variance assumptions of response variables before being subjected to repeated measurement analyses. Means were further separated by t-test if the overall null hypothesis of no significant difference between means at $\alpha = 0.05$ was rejected. Natural logarithm-transformed concentrations of individual soluble phenolics were analyzed by independent t tests between control and EAB treatments, separately for each observation time because many compounds were not consistently detected over the experimental period and thus data were not appropriate for repeated measures analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.07.003.

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