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Physiological responses of emerald ash borer larvae to feeding on different ash species reveal putative resistance mechanisms and insect counter-adaptations

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ABSTRACT

Emerald ash borer, *Agrilus planipennis* Fairmaire, an Asian wood-boring beetle, has devastated ash (*Fraxinus* spp.) trees in North American forests and landscapes since its discovery there in 2002. In this study, we collected living larvae from EAB-resistant Manchurian ash (*Fraxinus mandschurica*), and susceptible white (*Fraxinus americana*) and green (*Fraxinus pennsylvanica*) ash hosts, and quantified the activity and production of selected detoxification, digestive, and antioxidant enzymes. We hypothesized that differences in larval physiology could be used to infer resistance mechanisms of ash. We found no differences in cytochrome P450, glutathione-S-transferase, carboxylesterase, sulfotransferase, and tryptic BApNAase activities between larvae feeding on different hosts. Despite this, Manchurian ash-fed larvae produced a single isozyme of low electrophoretic mobility that was not produced in white or green ash-fed larvae. Additionally, larvae feeding on white and green ash produced two serine protease isozymes of high electrophoretic mobility that were not observed in Manchurian ash-fed larvae. We also found lower activity of β-glucosidase and higher activities of monoamine oxidase, *ortho*-quinone reductase, catalase, superoxide dismutase, and glutathione reductase in Manchurian ash-fed larvae compared to larvae that had fed on susceptible ash. A single isozyme was detected for both catalase and superoxide dismutase in all larval groups. The activities of the quinone-protective and antioxidant enzymes are consistent with the resistance phenotype of the host species, with the highest activities measured in larvae feeding on resistant Manchurian ash. We conclude that larvae feeding on Manchurian ash could be under quinone and oxidative stress, suggesting these may be potential mechanisms of resistance of Manchurian ash to EAB larvae, and that quinone-protective and antioxidant enzymes are important counter-adaptations of larvae for dealing with these resistance mechanisms.

1. Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive wood-boring insect introduced into North America from Asia, possibly during the early 1990s, where it is causing widespread mortality of ash (*Fraxinus* spp.) (Herms and McCullough, 2014). Recently, white fringetree, *Chionanthus virginicus* L. (Oleaceae), an ash relative, has also been documented as a larval host in North America (Cipollini, 2015). Larvae feed on the phloem, cambium, and outer sapwood layers, eventually girdling and killing susceptible hosts. Only a few studies have investigated mechanisms of resistance of angiosperm trees to wood-boring insects outside of the ash/EAB system (i.e. Dunn et al., 1990; Hanks et al., 1991, 1999; Muilenburg et al., 2011). This is especially concerning because of the potential economic and ecological impacts of exotic wood-borers (Aukema et al., 2010, 2011).

There is even less information available regarding physiological adaptations of wood-borers to counter host resistance mechanisms. Recent studies investigating the physiology, adaptations, and gene expression of phloem/xylem-feeding beetle species have made progress towards a better understanding of these systems (e.g. Crook et al., 2009; Geib et al., 2010; Scully et al., 2013, 2014). However, responses to feeding on different hosts are limited to a single study (i.e. Rajarapu, 2013). This author found that several glutathione-S-transferase (GST; EC 2.5.1.18) and cytochrome P450 monooxygenase (P450; EC 1.14.-.-) genes, as well

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as a β-glucosidase (EC 3.2.1.21) gene were expressed more highly in EAB larvae that had fed on green ash (*Fraxinus pennsylvanica*), a susceptible North American species, than those that fed on Manchurian ash (*Fraxinus mandschurica*), a resistant Asian species. Conversely, carboxylesterase (CarE; EC 3.1.1.1) and sulfotransferase (SULT; EC 2.8.2) genes, and genes associated with chitin metabolism, were more highly expressed in larvae that had fed on Manchurian ash.

Cytochromes P450 belong to an extremely important allelochemical detoxification enzyme family (Li et al., 2007), which oxidatively metabolize a wide variety of exogenous and endogenous substrates. GSTs are also major detoxification enzymes that have been shown to play a role in dietary tolerance of allelochemicals (Li et al., 2007). CarEs and SULTs also play detoxification roles (Li et al., 2007), and these genes were differentially upregulated in Manchurian ash-fed EAB larvae (Rajarapu, 2013). Rajarapu (2013) proposed that SULT contributes to detoxification of amines such as tyramine, which was found at greater concentrations in phloem of Manchurian ash relative to ash species more susceptible to EAB (Hill et al., 2012). Monoamine oxidases (MAOs) (EC 1.4.3.4) also metabolize tyramine, though MAOs have not been extensively studied outside their role in insect nervous systems (Sloley, 2004).

Faster browning (oxidation) rates of Manchurian ash phloem extracts, relative to EAB-susceptible ash species, have also been reported (Cipollini et al., 2011). Oxidation of phenolics produces toxic, reactive quinones that cross-link, denature, and reduce the quality of dietary proteins (e.g. Felton et al., 1992). This suggests that Manchurian ash may produce greater amounts of quinones or produce quinones more rapidly than susceptible ash species. However, EAB, like other insects, may be able to detoxify these quinones *via* quinone reductases (QRs; EC 1.6.99.2) that are induced by allelochemical consumption (Yu, 1987).

It has also been shown that EAB larvae differentially upregulate genes associated with digestion, including β-glucosidase, when feeding on susceptible green ash (Rajarapu, 2013). Several authors have reported reductions in the expression or activity of β-glucosidase in specialist insects feeding on host plants containing toxic glycosides (Pentzold et al., 2014), suggesting a potential adaptive mechanism aimed at decreasing the overall production of toxic products resulting from cleavage of the glucosidic bond. EAB may have this capacity since β-glucosidase genes were downregulated in larvae feeding on resistant Manchurian ash (Rajarapu, 2013), which contains several known phenolic glycosides (e.g. oleuropein and verbascoside) (see Whitehill et al., 2012, 2014).

Mittapalli et al. (2010) reported a high number of trypsin (a serine protease) and trypsin-like sequence domains in EAB larval midguts, but not other classes of proteases. This suggests that EAB is dependent on serine proteases (EC 3.4.21.-), and that interfering with them could be an effective host defense against EAB. Cipollini et al. (2011) and Whitehill et al. (2014) detected trypsin inhibitor activity in ash phloem extracts in radial diffusion assays, and Whitehill et al. (2014) tested the effects of soybean trypsin inhibitor (STI) on EAB larvae in bioassays with artificial diet. These authors reported that larval survival was not influenced at *in planta*-relevant trypsin inhibitor concentrations, though growth decreased in a dose-dependent manner. Ultimately, the relative importance of trypsin inhibitors as a mechanism of ash resistance to EAB needs further clarification.

Reactive oxygen species (ROS) of host origin can be highly damaging to insects, because they covalently bind to peritrophic membrane proteins or midgut cellular proteins and nucleic acids and cause lipid peroxidation (Bi and Felton, 1995). However, insect-produced antioxidant enzymes and free radical scavengers such as reduced glutathione (GSH) and ascorbate (Felton and Duffey, 1992) can protect herbivorous insects from ROS in their diet. Rajarapu et al. (2011) identified a superoxide dismutase (SOD; EC 1.15.1.1), a catalase (CAT; EC 1.11.1.6), and a glutathione peroxidase (GPX; EC 1.11.1.9) in EAB larvae. The high production of CAT in EAB larval midguts (Rajarapu, 2013) implies the presence of physiologically significant amounts of ingested H_2O_2 when feeding on ash phloem. GSH is an important electron donor in arthropods (Zhu-Salzman et al., 2008), acting as both an antioxidant and a co-substrate in enzymatically-driven antioxidant reactions. Glutathione reductase (GR; EC 1.8.1.7) reduces oxidized glutathione (GSSG) to GSH, regenerating it as an electron donor.

The goal of this study was to characterize the activities of detoxification, digestive, and antioxidant enzymes of EAB larvae when feeding on resistant Manchurian and susceptible white and green ash, which will improve understanding of resistance mechanisms of Manchurian ash to EAB, and the relative importance of larval physiological adaptations to these defenses. We predicted that enzyme activities of EAB larvae feeding on the resistant ash species reflect greater toxin exposure, as well as digestive and/or oxidative stress. Specifically, we predicted, based on previous gene expression experiments (Rajarapu, 2013), that larvae feeding on Manchurian ash would have higher CarE and SULT activities, and higher P450, GST, and β-glucosidase activities of larvae feeding on susceptible hosts. We also predicted that larvae feeding on Manchurian ash would have greater MAO activity because of the relatively high concentration of tyramine in Manchurian ash. Additionally, we predicted that the activity and production of trypsin isozymes would be influenced by unique trypsin inhibitors characteristic of the different ash species. Finally, we predicted that larval antioxidant enzyme activities and enzyme production would be greater in larvae feeding on Manchurian ash, due to the hypothesized ability of Manchurian ash to stress larvae via rapid oxidation of phenolics.

2. Materials and methods

2.1. Plants and insects

Larvae were obtained from two independent experiments, and differences in larval material utilized for enzyme analyses (i.e. age, instar, larval mass) reflect differences in experimental design. The experiment on responses of larvae to feeding on Manchurian and white ash was performed during the growing season of 2014, and the experiment on responses of larvae to feeding on green ash was performed during the growing season of 2013. For Manchurian ash-fed (Mf) and white ash-fed (Wf) larvae, 32 Manchurian ash (cv. 'Mancana') and 32 white ash (cv. 'Autumn Purple') trees (~2.5 cm basal diameter) were obtained from Bailey Nurseries, Inc. (Newport, MN), and grown outdoors in 58 L pots of mixed pine bark mulch and compost at the Ohio Agricultural Research and Development Center in Wooster, OH. Green ash-fed (Gf) larvae were collected from three replicate grafts of eight different green ash genotypes (total $n = 24$) that persisted in heavily EAB-infested natural areas in northeast Ohio and southwest Michigan. Green ash selections were propagated by grafting using either hot callus grafting (Carey et al., 2013) or bud grafting (Tubesing, 1987). Grafted trees were grown in an outdoor growing facility in 14.6 L containers in potting media consisting of Metro Mix® 510 (The Scotts Company, Marysville, OH) amended with 47 g Micromax Micronutrients (The Scotts Company, Marysville, OH), 376 g Osmocote® Plus 15–9–2 (The Scotts Company, Marysville, OH), and 700 g coarse perlite and 75 g aluminum sulfate per 2.8 cu. ft. bag. Potted green ash trees (2–3 years old, 1.5–2.5 m tall) were moved into a temperature-controlled greenhouse one week prior to inoculation.

EAB eggs were obtained from the USDA-APHIS-PPQ Biological Control Rearing Facility (Brighton, MI) (Mf and Wf larvae), or the