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Constitutive and Jasmonate-Inducible Defenses in Phloem of Two North American and Two Asian Ash Species Grown in a Common Garden

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CONSTITUTIVE AND JASMONATE-INDUCIBLE DEFENSES IN PHLOEM OF TWO NORTH AMERICAN AND TWO ASIAN ASH SPECIES GROWN IN A COMMON GARDEN

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY QIN WANG ENTITLED Constitutive and Jasmonate-inducible defenses in phloem of two North American and two Asian ash species grown in the common garden. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Wang, Qin. M.S. Department of Biological Sciences, Wright State University, 2009. Constitutive and jasmonate-inducible defenses in phloem of two North American and two Asian ash species grown in the common garden.

Emerald Ash Borer (EAB) is more damaging to North American ashes than Asian ashes. Variation in the resistance of ash species to feeding by larvae of EAB may be related to variation in levels of chemical defenses in the phloem. I compared constitutive and methyl jasmonate (MeJA)-inducible levels of several chemical defenses in the phloem of young Manchurian, Chinese, white, and green ashes. Manchurian ash is known to be highly resistant to attack by EAB in the field, while white and green ashes are both susceptible. The hypotheses of this experiment were that: (1) Manchurian (*Fraxinus mandshurica*) and Chinese ash (*Fraxinus chinensis*) trees' phloem or bark contains concentrations of unique secondary compounds and higher level of defense protein activities than white and green ash. (2) MeJA treatment will induce increases in content of protein, Peroxidase (POD), polyphenol oxidase (PPO) content and most of the secondary compounds in all species. Phloem extracts of Manchurian ash showed higher total soluble protein content and significantly faster browning than white and green ash, but lower peroxidase activity and polyphenoloxidase activity. Activities of PPO, POD, phenolic and lignin content, and the rate of browning reactions were not found to exhibit differences between MeJA treated and untreated groups. However, our HPLC results revealed Manchurian ash contained nine unique phenolic compounds: Homovanillic Alcohol, Esculetin, Esculin Related Cmpd, Esculin, Fraxin, Fraxidin hexoside, Pinoresinol dihexoside, Calceolarioside A, Calceolarioside B. We also found obvious increases in specific compounds (of the 20 compounds we reanalyzed in the four ash species) in Green ash, Chinese ash and Manchurian ash in the MeJA treated group. Also, Canonical Discriminant Function Analysis of five common compounds (Tyrosol Hexoside, Mandshurin, Pinoresinol compound, Syringaresinol and Oleuropein) revealed that Manchurian ash was different from the other three ashes, but closer to Green ash and Chinese ash. Green ash and Chinese ash were similar to each other. White ash was very

different from all other three ashes. In this study, the highly resistant Manchurian ash was distinguishable from the highly susceptible white ash by having higher total soluble protein content and a significantly faster browning. The susceptible green ash was distinguishable from Manchurian ash by having lower total protein levels and a lower degree of browning. The phloem chemistry of green and Chinese ash was largely similar in this study, indicating that they may be similar in their resistance to EAB, and White ash showed had levels of peroxidase activity, polyphenoloxidase activity, content of phenolic and lower rate of browning reaction than the other three species. From the result of Discriminate Function Analysis, White ash also showed maximal separation from the others. Since White ash is as susceptible as Green ash in North America, White ash may contain different compounds or enzymes than Green that may be attractive to EAB.

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INTRODUCTION

The invasion of Emerald Ash Borer to *Genus Fraxinus*

The ash genus *Fraxinus* is a common tree genus in North America deciduous forests. Various *Fraxinus* species are widely distributed across the eastern U.S, and portions of southeastern Canada, occurring in many different forest ecosystems (Little et al., 1953). *Fraxinus* species are also economically valuable. This wood can be used for bows, tool handles, and office furniture and sports equipment such as baseball bats, and they are important ornamental trees. However, in the late 1990s, a metallic wood-boring beetle (family Buprestidae), known as the emerald ash borer (*Agrilus planipennis*), has killed millions of trees in North America in less than 10 years, and continues to spread to northeastern North America and even Canada. In natural forests, EAB may dramatically change the composition and succession dynamics of many natural forests, and cause widespread damage to urban forests in the central and eastern U.S. (MacFarlane and Patterson Meyer, 2005).

Recent studies showed that an Asian ash species, Manchurian ash, is more resistant to EAB than North American congeners (Rebek at al. 2007). In China, EAB most likely attacks only stressed ash trees, including Manchurian ash (*F. manshurica*) (Gould et al. 2005). However, healthy North American ash species are susceptible to EAB, including white (*F. americana*) and green (*F. pennsylvanica*) ash, and attack usually leads to death. The reason EAB is rare in Asia may be related to their co-evolutionary history with Asian Ashes, which have evolved to resist them (Gould et al. 2005). Thus, we hypothesize that the compounds produced by Asian ash's phloem confer resistance to EAB because of their prior evolutionary history. In this study, I tested ten different proteins and

compounds among 4 different ash species from both Asia and North America. The ash species included: Chinese ash (*Fraxinus chinensis*), Manchurian ash (*F. manshurica*), white (*F. americana*) and green (*F. pennsylvanica*) ash. We predicted that Chinese ash and Manchurian ash would contain either higher levels of chemical defenses or a unique profile of defenses in the phloem compared to North American ashes, some of which are presumably associated with resistance to EAB.

OBJECTIVES

Hypotheses

My study focused on comparing constitutive and inducible levels of both defense protein activities and secondary compounds among different ash trees in the phloem, with and without MeJA treatment. My hypotheses are: (a) Manchurian (*Fraxinus mandshurica*) and Chinese ash (*Fraxinus chinensis*) trees' phloem or bark contains concentrations of unique secondary compounds and higher level of defense protein activities than white and green ash. (b) These defenses may be specially induced by MeJA and involved in resistance of Asian ashes to EAB. (c) MeJA treatment will induce increases in protein, Peroxidase, polyphenol oxidase, and most of the secondary compounds in all species.

Background on Emerald Ash Borer

Emerald Ash Borer (*Agrilus planipennis*) is an invasive insect, which arrived from eastern Asia countries through the process of importing merchandise shipped on solid wood packaging materials. This insect was first officially found in the Detroit, Michigan, U.S., area in the summer of 2002 (Herms et al. 2004). EAB is an invasive bark-feeding beetle that can kill healthy trees within a short period of time. This beetle normally lay eggs on stressed trees, but when the EAB populations increase, they will also attack healthy trees. As the result, younger ash trees can be killed with 1-2 years, and larger trees cannot survive longer than 4 years. This beetle lays its eggs in the region of the secondary phloem, where larvae create tunnels called galleries. The EAB adults cause little damage by chewing on ash leaves, but the larvae feed on the inner bark of ash trees, disrupting the tree's ability to transport nutrients, resulting in death for the tree. Currently,

the EAB has already infested and killed millions of ash trees (*Fraxinus* spp.) in Michigan, Ohio, Indiana, Maryland and Ontario (Herms et al. 2004) and has the potential to decimate all North American ash species. Tree removal and replacement cost would range between $1 billion and $4.2 billion in Ohio alone (Poland et al. 2006). The methods currently used to control EAB can be classified in three ways: physical control, biological control, and chemical treatment.

Control methods

A. Physical control

Physical control methods include removal and chipping of infested ash trees. This can only be effective before the infestations spread to nearby host plants. Small infestations in a localized area that are detected early may be eradicated easily, but more serious infestations cannot be controlled by this method because EAB adults can survive and spread from infested to uninfected areas by moving firewood (Mccullough et al.2007). Physical control is not a very efficient way to solve the problem.

B. Biological control

Biological control has been used for over 100 years in the U.S. and is considered to be a scientific and efficient way to control beetles. Recently in China, researchers found several natural enemies of EAB that may be useful as biological control agents in North America (Haack et al. 2002; Liu et al. 2003). *Spathius agrili* was found parasitizing up to 90 percent of EAB larvae in ash trees in China. Female *Spathius* parasitize EAB larvae by drilling through the bark and laying up to 20 eggs on its host. The hatching parasitoid

larvae feed and develop in the EAB larva, resulting it its death (Gould and Bauer 2007). *Tetrastichus planipennisi* and *Oobius agrili* also have been confirmed to successfully control EAB populations in northeastern Asia, which is the area of EAB introduction in China through surveys. However, candidate species found in China may not be used effectively against EAB in North America because of environmental differences such as optimum temperatures, relative humidity, and photoperiod. Finding a suitable natural enemy for eliminating EAB will require long -term studies.

C. Chemical control

A common method being used to prevent infestation of healthy trees from adult beetles presently is chemical control methods. Insecticides used for control of EAB fall into three categories: (1) systemic insecticides that are applied as soil injections or drenches; (2) systemic insecticides applied as trunk injections or trunk implants; and (3) protective cover sprays that are applied to the trunk, main branches, and foliage (Herms et al. 2007). However, chemical methods are more practical for preventing infestation of ash trees in urban areas than in natural areas because insecticides injections can be very expensive and time-consuming to apply. Moreover, research and experience have shown that best control will be obtained when treatments are initiated in the earliest stages of infestation before visible symptoms are present, or perhaps even the year before trees are infested. It is also important to realize that treatments will have to be repeated each year. In some cases, it may be more cost-effective to remove and replace the tree.

Resistance from Asian ash species

A recent study showed that an Asian ash species, Manchurian ash, is more resistant to the beetle than its North American congeners (Rebek et al. 2008). In common garden studies in the core infestation zone of EAB in Michigan, Green ash and white ash receive much more attack by EAB and are more likely to die from attack than Manchurian ash (Rebek et al. 2008). Larval EAB tend to make smaller feeding galleries on Manchurian ash than on susceptible North American ashes. Also, EAB has a faster development time in North America than Asia. The research from Wei et al. (2007) confirmed that EAB requires two years to complete one generation in Harbin, Heilongjiang Province (Yu, 1992) in Northeast of China, while EAB generally has a 1-year life cycle in southern Michigan (Mastro *et al.*, 2007), southern Ohio and the Maryland area. All the locations have the altitudes rang between 35°N-45°N and have a similar climate environment. Northeast of China also was the first place that life histories of EAB were documented. In Rebek et al.'s (2008) study, several different species of North American and Asian ash were compared for their susceptibility to EAB, including two native green ash cultivars, one native white ash cultivar, an Asian species, Manchurian ash, and an F1 hybrid cross between North American black ash (female) and Manchurian (male). The plantation was inoculated with the herbivore with ash logs infested with EAB. The results showed Manchurian ash had a much less damage and higher survival rate than North American ashes. The F1 hybrid had a low survival rate. This suggests that the F1 hybrid cultivar more closely aligns genetically with its susceptible North American parent relative to emerald ash borer resistance traits.

The reason EAB is less damaging in Asia may be related to their co-evolutionary history with Asian Ashes, which have evolved to resist them (Gould et al. 2005). Plants can evolve the ability to produce toxins that can reduce herbivre fitness when being attacked (Devendra and Bhavdish, 2008). Thus, we hypothesized that the compounds contained in Manchurian and Chinese ash phloem confer resistance to EAB because of their prior evolutionary history.

One common strategy for higher plants to defend against herbivores deterrence is through the wound-induced expression of plant defenses (Howe, 2004). Production of particular resistance phytochemicals is initiated by complex signal transduction pathways, which are related to specific signals at the site of tissue damage. The resistance phytochemical will change the plant's gene expression and metabolism and negatively affect herbivore growth and reproduction (Howe, 2004). Finding secondary compounds and proteins that confer resistance to EAB is the first step to development of new effective EAB control procedures.

Eyles et al. (2007) compared the secondary compounds in phloem from the main stem of white (*Fraxinus americana* L.), green (*Fraxinus pennsylvanica* Marsh.), and Manchurian ash (*Fraxinus mandshurica*). Manchurian ash was distinguished from the North American ash species by its unique content of hydroxycoumarins (particularly fraxin, mandshurin, and fraxidin hexoside), and significantly higher concentrations of pinoresinol glucoside and pinoresinol dihexoside (Eyles et al 2007). Many plant β-

glucosides are cyanogenic (Silva et al 2004); that means once being hydrolyzed by a β-glucosidase, the aglycone spontaneously generates cyanide. This may impair insect development by inhibiting trehalase, which is a key enzyme for controlling glucose availability in insects. Therefore, high concentrations of Pinoresinol glucoside may be a component of resistance in Manchurian ash that can affect protein digestibility by insects. A number of proteins in different ash species have also been investigated that have potential functions related to defense against EAB (Eyles et al. 2007). Cipollini (unpublished data) showed that Manchurian ash had high levels of trypsin inhibitor, polyphenoloxidase activity, and lower levels of peroxidase activity and total soluble protein content than white or green ash. Guaiacol peroxidase, polyphenoloxidase and trypsin inhibitor are often associated with induced resistance to both pathogens and insects (Cipollini et al 2004).

Methyl Jasmonic induced plant defenses

Jasmonic acid (JA) and its methyl ester, methyl-JA (MeJA) are members of the jasmonate family of oxylipins (Farmer and Ryan 1990; Farmer and Ryan 1992; Howe et al. 1996). Studies using MeJA treatment to induce bark defenses have made great progress. JA and MeJA both have major affects on the expression of wound-inducible proteinase inhibitors (PIs) and other defenses (Heil 2005). They are self-protection mechanisms to cope with herbivory. When induced by mechanical damage or insect attack, JA can increase anti-herbivore protein gene expression, like proteinase inhibitor and polyphenol oxidase (PPO) that inhibit the insect digestion process. Both proteinase

inhibitor and PPO can enhance the death rate of insect larva, or will reduce growth and survival of many insect herbivores (Heil 2005).

MATERIALS AND METHODS

Plants species and growth

Four-year-old saplings of Manchurian ash (*Fraxinus mandshurica*), Chinese ash (*Fraxinus chinensis*), White ash (*Fraxinus americana*) and Green ash (*Fraxinus pennsylvanica*) were randomly planted in a common garden on the campus of Wright State University in May 2007. There were 10 green ash (*Fraxinus pennsylvanica*), 11 white ash (*Fraxinus americana*), 8 Manchurian ash (*Fraxinus mandshurica*) and 10 Chinese ash (*Fraxinus chinensis*). Trees were spaced about 3 meters from each other, with mowed grass in between. All the trees were watered three times throughout the first growing season in addition to natural rainfall to promote establishment. All trees bore at least two branches that were useable (healthy and longer than 10 cm) for experiments. Trees were grown under these conditions for 14 months prior to their use in experiment.

Treatments-MeJA:

Methyl Jasmonic acid (MeJA) is a defense hormone that can induce increases in chemical defenses after wounding or the insect feeding process. On each tree, two size-matched branches were selected. One branch was treated by 100 mmol/L MeJA in water with 0.1% Tween 20; the matched branch was treated with only 0.1% Tween 20 for comparison. (Tween 20 is a common detergent, which is often used as a surfactant to solublize the MeJA and limit evaporation). On July 23, 2008, I applied 1ml of the solutions for each branch by using a medium sized paintbrush to help spread the solution evenly on the branch bark surface. MeJA solution was placed on a 10 cm long bark surface of treated branches. Two days later, I repeated the same treatment. Three days

later (07/25/2008) treated branches were cut from the trees. The samples were taken between 8cm and 10cm long, and included only the treated portion of the branch. For the samples from each tree, the treated and untreated branches were put in the same bag, which were separately labeled by using green and yellow stickers. At the end of the collection, I placed all the samples on dry ice, took them back to the lab and placed them -20^{o}C freezer for extraction later.

Measurements:

Defense protein extraction and analysis

Phloem samples of each species were obtained by dissecting branches using a razor blade, weighed (around 1g) and ground in liquid nitrogen. Soluble proteins were extracted from ground tissues in ice-cold 0.01M Sodium Phosphate buffer pH 6.8 containing 5% PVPP. Homogenates were centrifuged for 10 min at 7,000 x g, and the cleared supernatants were transferred to fresh tubes. For precipitating most soluble proteins, acetone precipitation was performed following the extraction. Ten ml of cold acetone was added to 2 ml of extract and well vortexed. Tubes were placed in a freezer for 30 mins. After centrifugation at 7000 rpm for 10 min, the supernatant was poured out and the pellet was kept, and air dried for 30 mins. Then pellet was resuspended in 1.5 ml of 0.02 M Naphos buffer pH 6.8, by vortexing, and extracts were stored at -20^{o}C until they were analyzed.

Polyphenol oxidase, peroxidase, and trypsin inhibitor activities were assessed in this experiment. The methods were conducted as in Moran & Cipollini (1998).

Polyphenoloxidase activity was determined by the following procedures; I set up and labeled a standard micoplate (96-well plate). The first column was filled with blanks and the rest of the wells were for samples (in duplicate). I added 40ul of NaPhos buffer for blanks. For the sample wells, each contained 0.030ml extract and 0.00294 M caffeic acid in 0.05M sodium phosphate buffer, pH 8.0. I kept the micoplate on ice while loading samples. Samples were analyzed by using a microplate reader. Polyphenol oxidase activity was calculated as the change in absorbance at 470nm per minute per milligram protein, expressed as ΔAbs_{470nm} x $minute^{-1}$ x mg extract $protein^{-1}$ (Cipollini and Redman 1999). Peroxidase activity was assayed using a similar assay, with guaiacol and hydrogen peroxide as substrates. 0.015 mL protein extract and 0.015ml 25% guaiacol with pH 6.0 were used as the substrate in 0.01 M Na-Phosphate Buffer, pH 6.0. and the change in absorbance at 470 nm per min per milligram of protein was followed in the microplate reader (Cipollini 1998). Trypsin inhibitor activity was assayed using the agar plate diffusion technique described in Cipollini and Bergelson (2000). Phloem extracts were loaded into wells in an agar plate supplemented with trypsin. The plate was then stained to show unbound trypsin after 18 hours of diffusion. The diameter of clear halos around the wells was correlated with the amount of trypsin inhibitor using a standard curve. Trypsin inhibitor contents was expressed as μg trypsin inhibitor x mg extract $protein^{-1}$ and μg trypsin inhibitor x g $FW^{-1.}$.

New extracts were made only for browning rate analysis. The procedure was similar to that for protein analysis except not containing PVPP in the buffer. The browning reaction was examined immediately after removal of supernatants from the centrifuged

phloem homogenates. 0.3 mL aliquots of each soluble extract were placed in a standard micoplate (96-well plate) and the absorbance at 470nm was recorded twice: before and after 1 hr incubation at room temperature. The browning reaction could be observed within a few minutes around wounded phloem tissues of ash, and is driven in plant tissues primarily by the action of phenol oxidases (including both peroxidase and polyphenol oxidase) on endogenous phenolics resulting in quinone formation and subsequent polymerization (Cheng and Chrisosto 1995, Dehon et al. 2002). We propose that higher rate of browning reaction may relate to a stronger self-healing ability. The rate of browning reaction was expressed as $\Delta Abs_{470nm} \times hr^{-1} \times$ mg extract protein^{-1} and $\Delta Abs_{470nm} \times hr^{-1} \times g\ FW^{-1}$.

Total protein of all extracts was measured using bovine serum albumin as a standard and bio-Rad protein dye reagent as described in Bradford (1976). All assays were performed in duplicate.

Ash soluble Phenolic and Lignin Extractio

Phloem samples of each species were weighed to 100mg, ground in liquid nitrogen, and extracted twice with 500 ML of HPLC grade 100% methanol (Fisher, Pittsburgh, PA) for 48 hour in the dark at 4^{o}C. Extracts were centrifuged for 5 min at high speed (13400rcf). Supernatants were used for total phenolics, and the pellet was used for lignin assays. Phenolic content was analyzed by using the Folin method of Bonello and Pearce (1993). Two µL of methanol extract were added to 75 µL of methanol and 500 µL of water and mixed, then 37.5 µL of Folin's Phenol Reagent were added (Sigma, St. Louis,

MO). Three minutes later I added 37.5 µL of 1 M $NaHCO_3$ and mixed thoroughly. After one hour incubation at room temperature, the samples were analyzed spectrophotometrically against a standard curve of gallic acid at 725 nm. Phloem tissue was extracted twice in 500 ul of HPLC grade methanol (Fisher, Pittsburg) for 24h at 4°C. The supernatants were stored at -20°C till they were analyzed by HPLC. HPLC analysis was conducted as in Eyles et al (2007).

The procedures for lignin measurement were as described by Bonello and Blodgett (2003, 2007) were followed with slight modifications. In brief, the pellets for phenolic extraction were washed with 1 ml of water and 0.9 ml of *tert*-butyl methyl ether (Sigma). The pellets were left to dry overnight, and then processed according to the methods of Bonello and Peace (1993). In brief, after the addition of 2 ml of 0.5 M aqueous NaOH to the pellet, the suspension was heated in a stoppered tube for 16 h at 80 C. After cooling, the residue was centrifuged, 1.6 ml of the supernatant was taken, adjusted to pH 7.9 with 2.5 M HCI, and made up to 2.5 ml with water. Following this, 0.5 ml of the solution was diluted with 2.5 ml of 0.06 M aqueous NaOH. The UV spectrum of this last fraction (pH 12) was determined against that of the fraction at pH 7.9 using a Varian spectrophotometer (Series 634; Varian Ltd, Walton-on- Thames, UK) connected to a Varian recorder (Model 9176). The spectrum was run between 220 and 400 nm.

Identification of phenolic secondary compounds:

Compounds were identified by HPLC as in Eyles et al. (2007). The graphs produced from the HPLC have peaks that correspond to specific soluble compounds. Peaks

showing significant concentrations were targeted. I selected 20 major peaks for analysis that matched up to the compounds already identified in ash species in Eyles et al, 2007. Concentrations of each compound were determined using peak areas.

Statistical Analysis:

The program SAS was used for statistical analysis. Nested ANOVA was used for statistical analysis, and defense protein activities total protein content, lignin and the browning reaction were compared among four species and among methyl jasmonate treatments. Two factors in the model included species and MeJA nested within species. Since I measured multiple attributes of the same individual trees, P-value for multiple comparisions were adjusted by using a Bonferroni correction. We found no significant differences between treated and untreated groups, so we redid the analysis by using one way ANOVA with species as the independent variable. Canonical discriminant analysis (CDA) was used to determine whether differences in plants phenolic chemical levels separate all four species. Canonical discriminant analysis (CDA) is a multivariate statistical procedure (Campbell and Atchley, 1981). CDA permits comparison of the degree of variability existing and simultaneously examines differences in the chemical variables and indicates the relative contribution of each variable to discrimination among species. Five phenolic compounds that all ash species contained were used as variables for the analysis. I used the PROC CANDISC procedure, with species as the factor. Log transformation was applied as needed to meet the assumptions of normality.

RESULTS

MeJA had no discernable effect on levels of peroxidase, polyphenoloxidase and Trypsin inhibitor activities or on phenolics, lignin and browning reaction. Therefore, all analyses of these compounds focused on differences between species.

Peroxidase activities

Fig 1A (Activity per unit extract protein) and Fig 1B (Activity per unit fresh mass) both showed that differences in POD activities among species was significant (Table 1). Green ash and Chinese ash showed higher peroxidase activity, white ash had the lowest activity per unit protein (Fig 1A). Manchurian ash was intermediate. In Fig B, the result also shows Green ash had the highest peroxidase activities, while Manchurian ash had similar but lower activity than Green ash (Fig 1B). White ash was still the lowest. Peroxidase activity of Chinese ash is lower than Green ash and Manchurian ash, but much higher than White ash.

Polyphenoloxidase activity

It showed some differences in both per unit Protein and per unit mass. Chinese ash and Green ash had similar high polyphenoloxidase activities (Fig 2A). White ash and Manchurian ash had lower polyphenoloxidase activities (Fig 2A). When analyzed permit mass, Chinese ash and Green ash were still had the same level of polyphenoloxidase activities, and White ash and Manchurian ash had lower polyphenoloxidase activities. One way ANOVA (Table 1) results showed polyphenoloxidase activities showed

significant difference among species relative to protein, but there is no significant difference relative to mass (Table 1).

Soluble phenolic concentration

Chinese ash and Green ash were similar and relatively high. Manchurian ash was lower, and white ash had the lowest level. Significant differences in phenolics activities were found among all ash species (Table 1).

Total amount of lignin

There was no significant difference in total lignin among species. (Fig 4), (Table1).

Trypsin inhibitor activity

Chinese ash had the highest trypsin inhibitor activities per unit protein (Fig 5A), Green ash was slightly lower than Chinese ash, but not different. Manchuian ash trypsin inhibitor activities were the lowest but not different than White ash. For the per unit mass, analysis no significant effects among species in trypsin inhibitor activities was observed. (Fig 5B), (Table 1).

Protein content

Manchurian ash showed the highest amount of total protein (Fig 6). Green ash, Chinese ash and White ash were at the similar level, and lower than Manchurian ash. There was a significant difference the amount of total protein among species (Table 1).

Rate of browning reaction

Manchurian ash was found to have much higher browning reaction rate than white ash (Fig 7). Green ash was a little lower than Manchurian ash, but similar. Chinese ash was intermediate. One way AVOVA data showed there was significant differences among species (Table 1).

HPLC profiles

Table 2 and 3 shows the 20 compounds that were analyzed in the 4 ash species. They share five compounds: 3.(Tyrosol Hexoside), 10.(Mandshurin), 12.(Pinoresinol compound), 14.(Syringaresinol) and 19.(Oleuropein). Manchurian ash in particular contained nine unique compounds from the other 3 species, 2. (Homovanillic Alcohol), 4. (Esculin), 5. (Esculin Related Cmpd), 7. (Esculin), 8. (Fraxin), 9. (Fraxidin hexoside), 11.(Pinoresinol dihexoside), 15. (Calceolarioside A), 18. (Calceolarioside B). Also, compounds 6.(Syringin/Oleoside), 13.(Pinoresinol Glucoside), 16.(Oleuropein hexoside), 20. (Ligustroside) were found in the other 3 ash species but were not present in Manchurian ash. There was no obvious evidence of MEJA treatment induction found in MeJA treated group.

Result of the Canonical discriminant analysis

The centroid values for the five compounds canonical discriminate functions for the four cultivars were plotted (Figure 12). We analyzed five different compounds the four ash species all share, including Tyrosol Hexoside, Mandshurin, Pinoresinol compound,

Syringaresinol and Oleuropein. The distribution showed Manchurian ash was far different from the other three ashes, spread in the right upper side of the graph. Chinese ash and Green ash cluster closely to each other on the middle right side. White ash is maximally separated from the others, gathered around at the left side of the graph. The first three canonical variates were significant (Table 4) ($P< 0.01$). Each canonical variate is the linear combination of the independent variables (traits). Canonical correlation measures the strength of the overall relationship between canonical discriminant variate and five compounds for all four different ash species. The significant canonical correlation between five common compounds and the first canonical variate ($r = 0.86$), compounds and second canonical variate ($r = 0.63$) and compounds and third canonical variate ($r = 0.52$) indicates that the canonical variates can explain the differentiation of the species on the basis of five common compounds. Also, the data from the first canonical correlation (table 4) showed Esculetin had the largest Canonical loading on the first two discriminant variates. Asian ash and North American ash are different in not only containing unique compounds, but also for the compounds they share; there are significant differences in the quantities for each species.

DISCUSSION

The objective of this experiment was to determine if Manchurian ash, which has higher resistance to EAB than North American ashes, may have unique or dramatic differences in the amount of defensive compounds and proteins than North American ash species. From our results, Manchurian ash did show higher level of protein content per fresh mass and higher rate of browning reaction than the other species.

I hypothesized that Manchurian and Chinese ash would have higher levels of defensive protein activity than White and Green ash. Manchurian ash did not exhibit higher activities level of PPO and POD. However, Chinese ash and Green ash both had higher and similar levels of activity of PPO and POD. Chinese ash and Green ash are widely separated in distribution: *Fraxinus pennsylvanica* (Green ash) is distributed in the central and eastern USA, Canada, and *Fraxinus chinensis* (Chinese ash) is found in Korea and northern China. According to a phylogenetic reconstruction of the genus *Fraxinus* based on the DNA sequences from 106 nuclear ribosomal ITS data (Fig 13.)(Wallander, 2008), Fraxinus pennsylvanica (Green ash) and Fraxinus chinensis (Chinese ash) are widely separated in two different clades. The fact that these two ash species had such similar defense activities cannot be explained by their phylogenetic relationship. It is possible that our cultivar of Chinese Ash was actually misidentified and actually represents a Green ash or Green x Chinese hybrid, but we lack additional evidence in support of this hypothesis. Manchurian ash did stand out on the rate of browning reaction and total protein content. Chinese ash and Green ash had a similar level and white ash

had the lowest rate of browning reaction. This matches the fact that Manchurian ash is more resistant to EAB.

The four ash species were also treated with MeJA to see if it had any effect on activities of the defense proteins and other traits. Our analysis showed there was no significant effect of this treatment on any ash species. This result didn't support our hypothesis that ash samples with MeJA treatment should have much higher protein and enzymes activities than our control ones. This result may have been caused by low concentration of MeJA (100 mM MeJA with 0.1% Tween-20 detergent in10L distilled water), which we used based on previous studies used on pine trees (Heijarial, et al.2008). In Heijarial's study, the MeJA solution was used at the same concentration as we mentioned above. They applied treatment solutions over the whole tree including needles, branches, and bark of the trunk by using a handheld sprayer. Treatments were given three times during two weeks. Surrounding trees were protected from the spray mist with a portable plastic screen. Our treatment was only placed on small sections of selected matched branches and solutions were applied two times within one week. In Heijarial's study, needle chemistry was tested, including: monoterpenes, sesquiterpenes, and tricyclic resin acids. The result showed MeJA treatment causes increases of monoterpenes and sesquiterpenes in certain young pine tree species (Heijarial, et al.2008). One possible reason for our unexpected results might be that different tree species have different response to MeJA.

In our analysis, White ash showed distinct results compared to the other North American species. For instance, it has consistently low levels of POD activities, low soluble phenolic concentration and a low rate of the browning reaction. There is no evidence that white ash cultivars are any more susceptible or more attractive to EAB than other North American ash species.

The mechanisms of resistance in ash species to insects are largely unknown. Our HPLC analysis focused on 20 compounds based on preliminary data (Whitehill, personal communication). The four ash species contained several different compounds. Manchurian ash had nine compounds not found in the other species. They were Homovanillic Alcohol, Esculin, Esculin Related Cmpd, Esculin, Fraxin, Fraxidin hexoside, Pinoresinol dihexoside, Calceolarioside A, Calceolarioside B. It also contained a particularly high amount of Syringin/Oleoside, Pinoresinol Glucoside, Oleuropein hexoside and Ligustroside. Our result also show that Chinese ash and Green ash have more similarity in protein and enzyme activities, and many common compounds. In Eyles et al. (2007), phenolic profiles of young Manchurian, white, and green ashes were examined. Their HPLC result was consistent with ours and revealed a unique content of fraxin, fraxidin hexoside, calceoloariosides A and B and significantly higher concentrations of Pinoresinol Glucoside in Manchurian ash.

Esculin has been suggested by Silva et al. (2006) to retard insect development by inhibiting a major enzyme which controls glucose availability in insects. Pinoresinol, which is one of the simplest lignins, also was detected in a significantly higher amount in Manchurian ash (Eyles et al. 2007). Some lignan members have been shown to have

antifeedant activity and can inhibit larval growth of bloodsucking bug (*Cimex lectularius*) and milkweed bug (*Oncopeltus fasciatus*) (Cabral et al. 1999; Garcia et al. 2000).

Buschmann et al. (2002) found out that hydroxycoumarins play an important role in plants wounding responses, and induces the production of defensive compounds and enzymes. Hydroxycoumarins (1 esculin, 2 scopolin, 3 esculetin, 4 scopoletin) are found in the cassava root, which were proved by Rodriguez et al. (2000) to have possible functions as anti-microbial activities against a variety of different fungal and bacterial organisms. Other unique compounds of Manchurian ash such as Homovanillic Alcohol, Fraxin, Fraxidin hexoside, Calceolarioside A, and Calceolarioside B have not been examined for their bioactivity.

We found that there was a significant increase in some compounds after MeJA treatment in three ash species. For Green ash, ten compounds we detected to have increased after the treatment. In Manchurian ash, eleven compounds were found to exhibit dramatic increases out of sixteen. In Chinese ash, six compounds increased. There was no significant increase compounds in White ash. This result implies MeJA-treatment has a positive effect by inducing certain defense compounds on Manchurian ash, Green ash and Chinese ash, but not several other traits that we measured.

Although our result didn't show differences in lignin content among species, lignin is known to perform a leading role in induced defensive reactions on living trees (e.g. Akai and Fukutomi 1980; Wainhouse et al.1990). Many previous studies have showed that

lignin is one of the most important defensive chemicals in plants defense against fungi (e.g. Akai and Fukutomi 1980). It has also been shown that the presence of lignin in bark can affect bark feeding and beetle tunneling (Wainhouse et al., 1990). Lignin reduces damage by spruce bark beetle (*Dendroctonus mican*) (Wainhouse et al., 1990). Spruce bark beetle is also an invasive insect, which was accidently introduced to Britain from mainland Europe (Bevan and King 1983). Now it is widely distributed in spruce plantations in Wales and the west Midlands. Lignin reduced larval survival, growth rate, and weight (Wainhouse et al., 1990). At the same time, a number of other significant changes were induced by wounding, including an increase in the concentration of nitrogen and decreases in the moisture content and the concentration of free sugars (Wainhouse et al. 1998). Similar factors may also be important for ash resistance to EAB.

Overall, we compared Asian ash and North American ash species among different factors. Significant differences in the chemical defense were found between resistant ash species (Manchurian ash) and North America ash. MeJA treatment was applied to all four ash species. Activities of PPO, POD, content of phenolic and lignin, the rate of browning reactions were not found to exhibit differences between treated and untreated groups. Whether defense chemicals were induced by MeJA on ash trees was unclear since no clear differences was found between treated and untreated groups on activities of defense protein, but there were clearly positive effects on some compounds from our HPLC data. We found increases of specific compounds in Green ash, Chinese ash and Manchurian ash. The fact that Manchurian ash has more resistance to EAB may be related to particular defenses being induced in Manchurian ash to EAB attack. Whether Asian ash

trees could produce certain chemical compounds, which has a strong influence on EAB is still unknown. A number of studies suggest that North American ash abundance is a positively associated with the disturbance in the landscape (Fowells, 1965; Taylor, 1971; Arevalo et al., 2000; Lesica, 2001; Battaglia et al., 2002). North American ashes are an important component of many forest ecosystems and have been put at substantial risk from the introduction of EAB. Further studies are needed to examine the mechanistic basis of resistance to EAB and effects of MeJA on ash tree growth.

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Table 1 Result of a one way ANOVA several defense related chemical traits in four ash species. (P-value was adjusted for multiple comparisons by using a Bonferroni correction)

Factor	POD/prot	POD/mass	PPO/prot	PPO/mass	Phenolic	Prot/mass	lignin	BR	TI/prot	TI/mass
F value	3.03	3.72	3.92	1.74	3.585	3.12	0.60	4.65	2.68	1.73
(P)	(0.00077)	(0.00018)	(0.00011)	(0.011)	(0.0002)	(0.00064)	(0.076)	<0.0001	(0.0016)	(0.011)
DF	7, 70	7, 70	7, 70	7, 70	7, 70	7, 70	7, 70	7, 70	7, 69	7, 69

POD/prot: per unit extracts protein. POD/mass: per unit fresh mass. PPO/prot: PPO/mass: Phenolic: Prot/mass: lignin: BR: TI/prot : TI/mass:

Table 2. List of phenolic compounds that were examined in ash phloem samples by HPLC.

1	Hydroxytyrosol hexoside	11	Pinoresinol dihexoside
2	Homovanillic Alcohol	12	Pinoresinol compound
3	Tyrosol Hexoside	13	Pinoresinol Glucoside
4	Esculetin	14	Syringaresinol
5	Esculin Related Cmpd	15	Calceolarioside A
6	Syringin/Oleoside	16	Oleuropein hexoside
7	Esculin	17	Verbascoside
8	Fraxin	18	Calceolarioside B
9	Fraxidin hexoside	19	Oleuropein
10	Mandshurin	20	Ligustroside

Table 3. Mean peak areas (±SE) of selected individual phenolic compounds in white, green, Chinese and Manchurian ash in control and MeJA treated groups

Compounds	White		Green		Chinese		Man	
	Con mean (SE)	Trt mean(SE)	Con mean(SE)	Trt mean(SE)	Con mean(SE)	Trt mean(SE)	Con mean(SE)	Trt mean(SE)
1	6.1 (1.73)	4.40 (1.07)	–	–	1.49 (0.24)	1.75 (0.39)	8.30 (1.91)	6.17 (1.40)
2	–	–	–	–	–	–	3.94 (5.33)	4.95 (7.74.)
3	1.73 (0.63)	1.62 (0.23)	2.02 (2.88)	3.39 (7.73)	2.12 (3.21)	1.66 (0.28)	1.98 (0.18)	1.97 (0.21)
4	–	–	–	–	–	–	3.11 (5.46)	5.07 (5.82)
5	–	–	–	–	–	–	13.57 (9.69)	16.18 (10.94)
6	9.29 (1.83)	8.28 (0.83)	13.85 (13.42)	14.04 (13.55)	11.4 (12.6)	11.63 (10.87)	–	–
7	–	–	–	–	–	–	10.28 (1.61)	48.37 (17.79)
8	–	–	–	–	–	–	49.5 (7.58)	50.27 (7.0)
9	–	–	–	–	–	–	30.23 (4.09)	35.0 (4.87)
10	1.77 (1.63)	1.54 (2.14)	32.43 (8.19)	32.69 (8.20)	20.26 (3.03)	14.59 (2.63)	20.52 (3.19)	20.75 (3.2)

(Table 3 continued)

11	–	–	–	–	–	–	3.25 (0.50)	3.39 (0.44)
12	8.683 (1.15)	9.9984 (1.87)	0.88 (1.27)	0.97 (2.30)	3.75 (0.74)	3.35 (0.56)	7.81 (1.55)	7.21 (1.28)
13	5.84 (1.08)	4.15 (0.94)	5.82 (2.31)	7.45 (2.5)	4.89 (1.06)	5.6 (8.12)	–	–
14	18.33 (3.15)	14.27 (2.53)	9.14 (4.9)	9.97 (2.6)	7.61 (1.5)	11.16 (2.24)	35.05 (5.34)	42.76 (9.06)
15	–	–	–	–	–	–	35.19 (13.67)	38.1 (20.65)
16	6.48 (1.52)	4.55 (8.36)	5.87 (2.09)	8.68 (2.1)	5.27 (1.18)	4.23 (8.7)	–	–
17	2.67 (5.31)	2.04 (2.03)	1.68 (3.96)	1.80 (4.05)	10.84 (3.96)	6.44 (2.15)	3.70 (1.28)	5.0 (3.48)
18	–	–	–	–	–	–	17.15 (6.0)	18.0 (6.75)
19	1.22 (3.40)	0.92 (0.37)	2.25 (8.82)	2.22 (3.33)	5.59 (1.41)	7.60 (2.80)	3.11 (7.67)	4.0 (1.14)
20	12.75 (1.89)	15.51 (2.33)	13.81 (3.02)	14.0 (2.87)	8.08 (2.29)	10.79 (2.08)	–	–

(Contents expressed in peak areas)

Table4. Canonical loadings of the independent variables on five common compounds among 4 ash species.

Trait	Canonical discriminant variate		
	1	2	3
Homovanillic Alcohol	1.43	-0.46	-1.28
Esculetin	3.79	1.19	-0.07
Esculin Related Cmpd	-1.73	1.86	1.07
Esculin	-1.59	-0.84	1.29
Fraxidin hexoside	1.31	-0.99	1.7
Canonical correlation	0.86	0.63	0.52
F-value	15.42	8.94	8.81
P level of significance	<0.001	<0.001	<0.001
DF	15193	8142	372
Variance accounted for, %	0.73	0.17	0.09

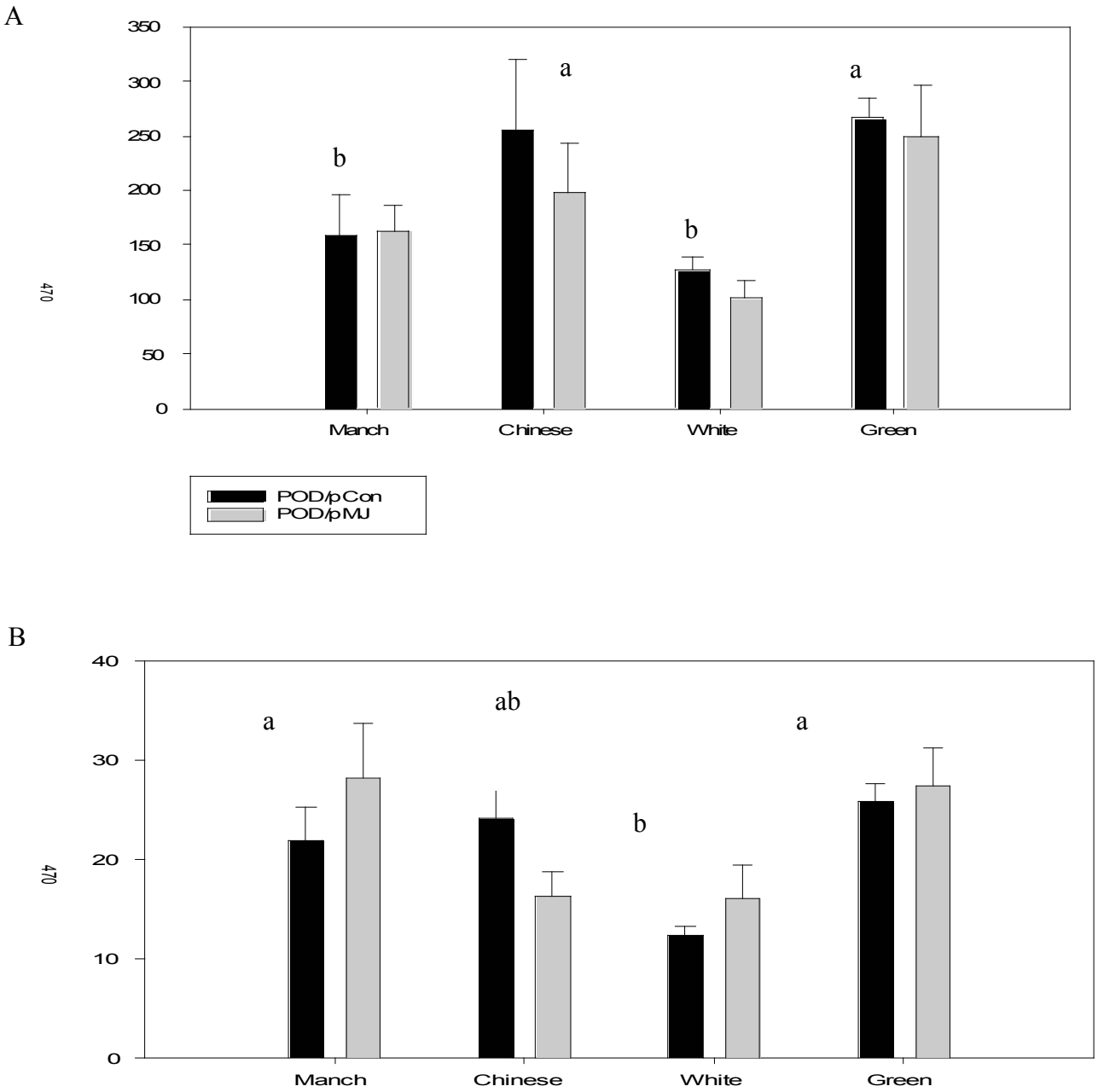

Figure. 1 Constitutive and MeJA- inducible peroxidase activities in phloem extracts of Manchurian, Chinese, white, and green ash. A. Activity per unit extracts protein B. Activity per unit fresh mass. Each bar represents the mean (SE) of ten replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

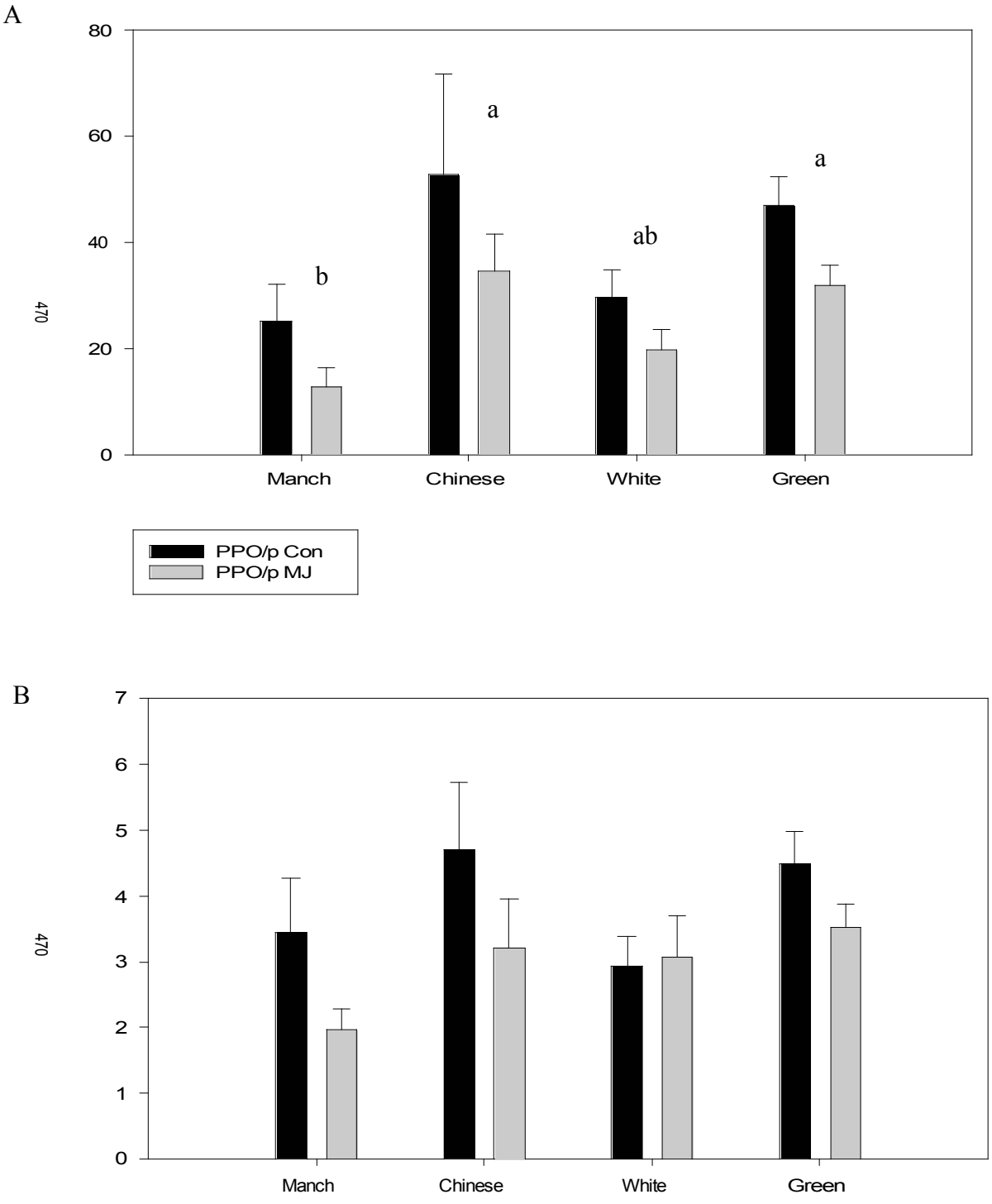

Figure 2. Constitutive and MeJA- inducible polyphenoloxidase activity in phloem extracts of Manchurian, Chinese, white, and green ash. A. Activity per unit extracts protein B. Activity per unit fresh mass. Each bar represents the mean (SE) of ten replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

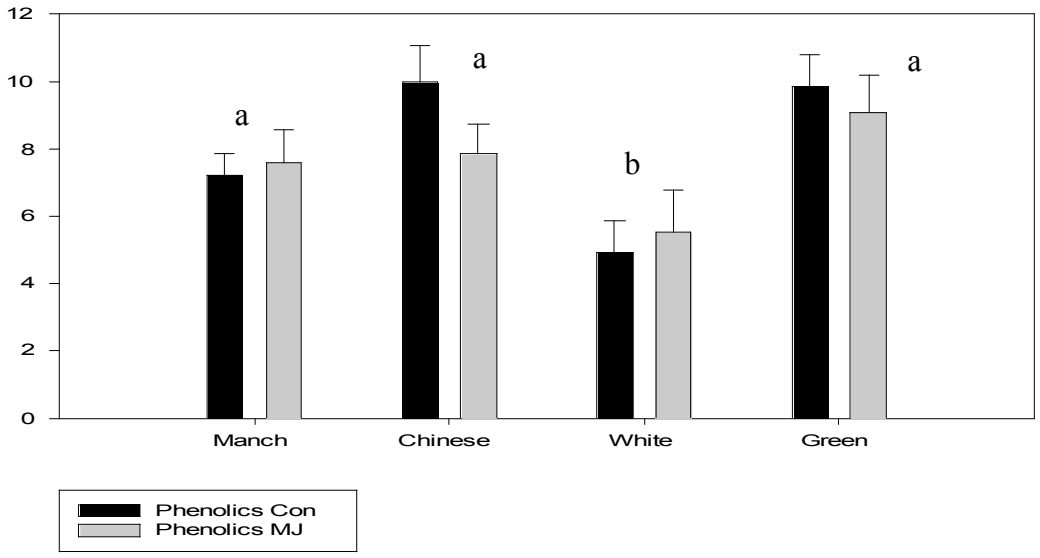

Figure 3. Constitutive and MeJA-inducible soluble Phenolic concentration in phloem extracts of Manchurian, Chinese, white, and green ash. Activity per unit fresh mass. Each bar represents the mean (SE) of ten replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

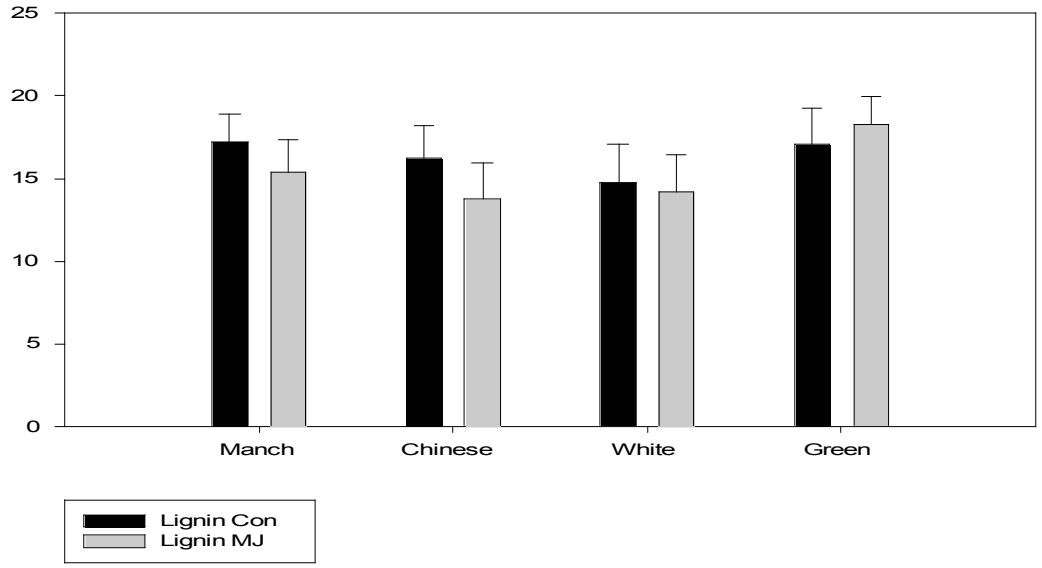

Figure 4. Constitutive and MeJA-inducible total Lignin in phloem extracts of Manchurian, Chinese, white, and green ash. Activity per unit fresh mass. Each bar represents the mean (SE) of eight replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

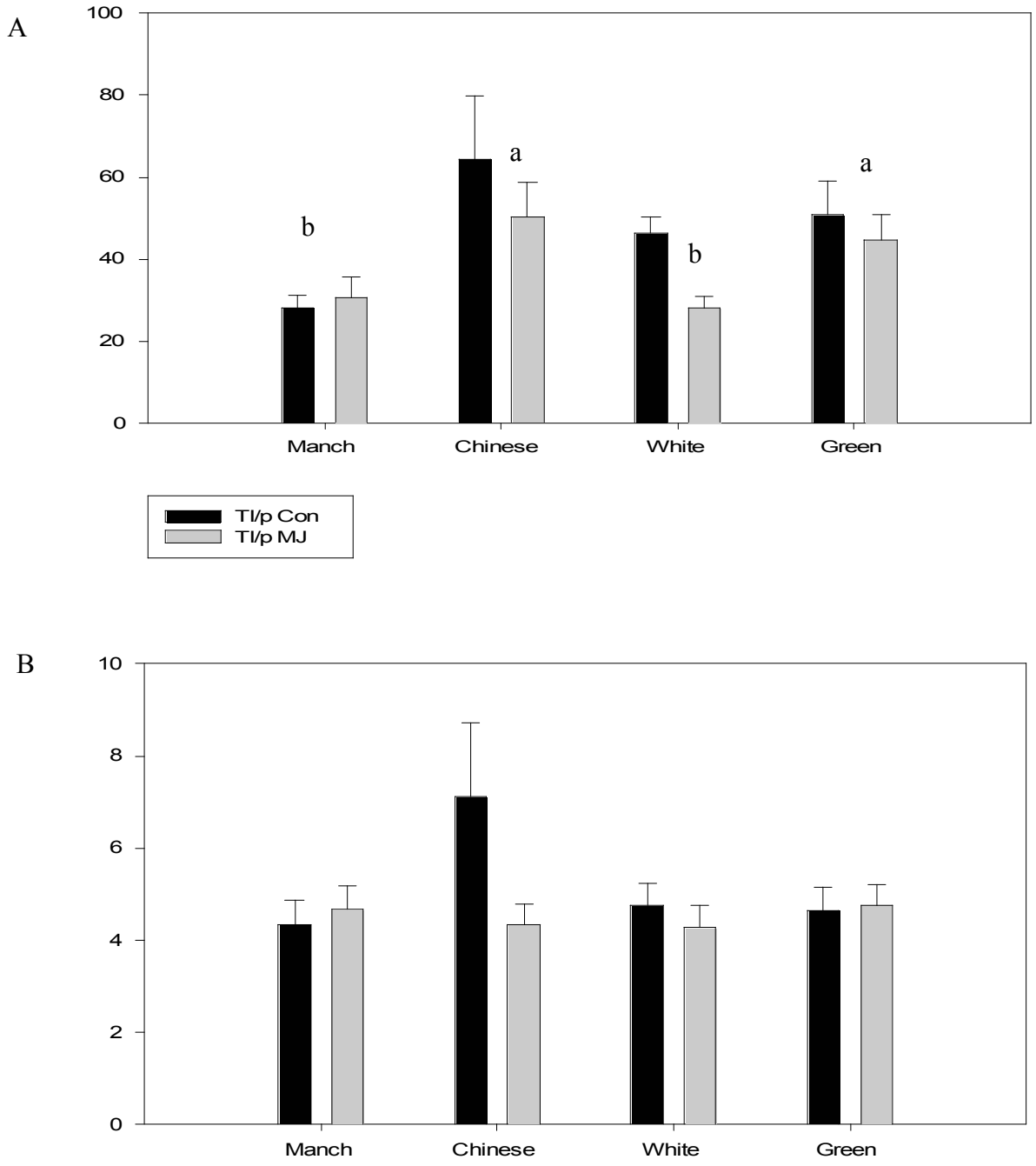

Figure.5 Constitutive and MeJA- inducible trypsin inhibitor activity in phloem extracts of Manchurian, Chinese, white, and green ash. A. Activity per unit extracts protein. B. Activity per unit fresh mass. Each bar represents the mean (SE) of ten replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

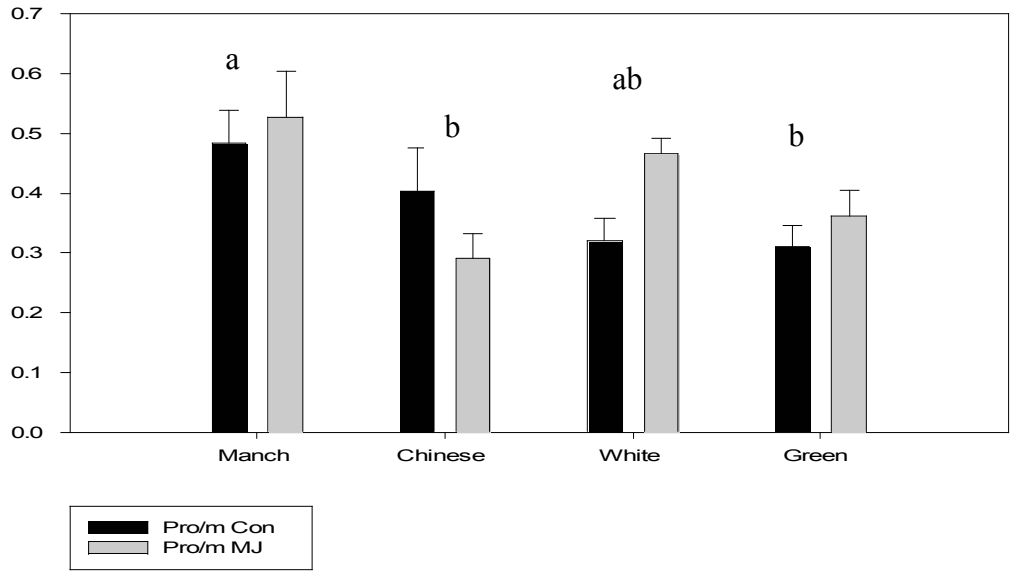

Figure. 6 Constitutive and MeJA- inducible soluble protein content in phloem extracts of Manchurian, Chinese, white, and green ash. Activity per unit fresh mass. Each bar represents the mean (SE) of ten replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$.

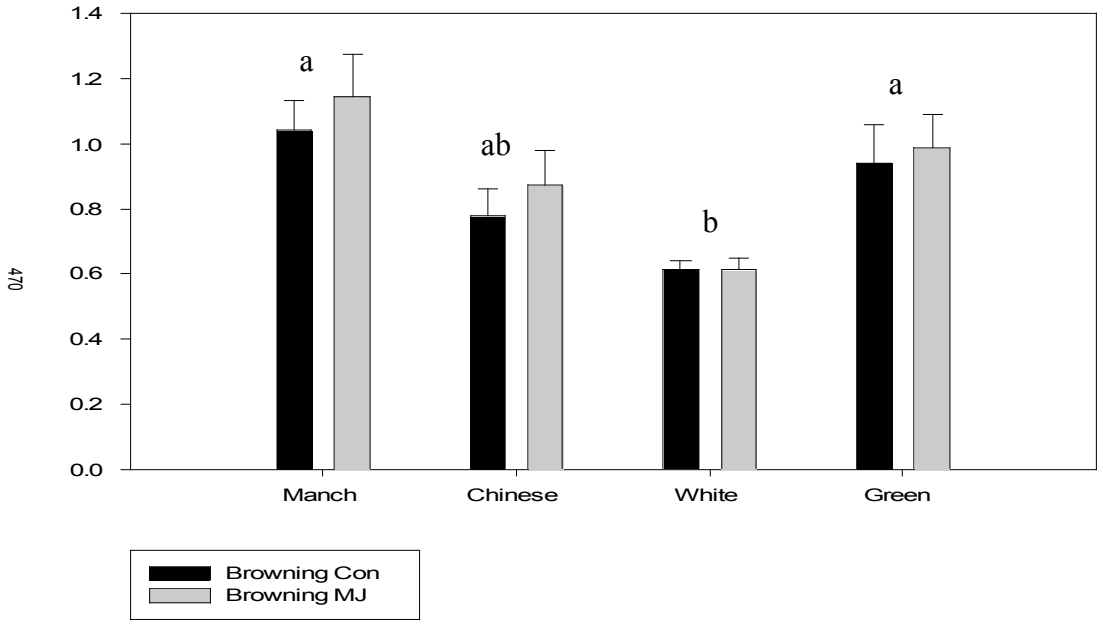

Figure. 7 Constitutive and MeJA- inducible rate of browning reaction for phloem extracts of Manchurian,Chinese, white, and green ash. Rate of browning reaction per unit fresh mass Each bar represents the mean (SE) of eight replicate plants. Bars with different letters for each species are significantly different at $P \leq 0.05$

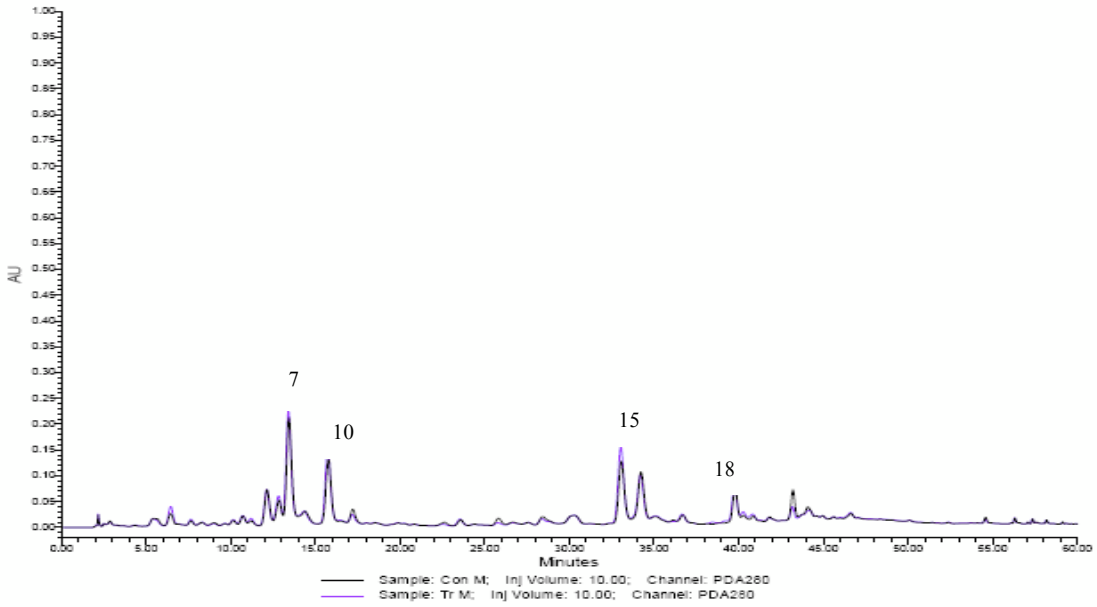

Figure 8. Comparison of HPLC profiles at 280 nm of constitutive phloem extracts (100% methanol) of two Manchurian Ash groups: treated and control groups overlay. For peak assignments of the major peaks, see Tables 2 and 3

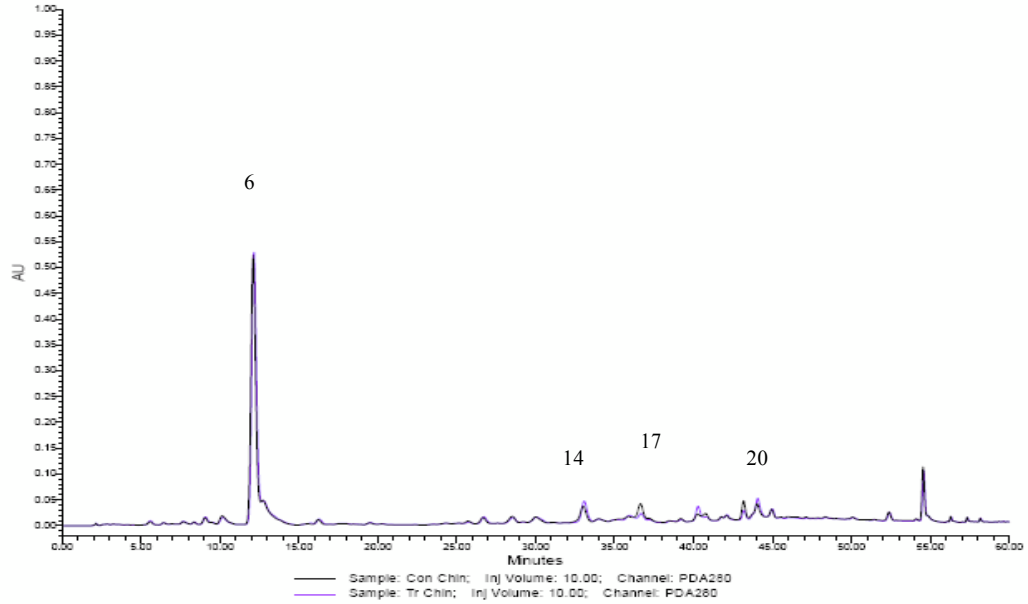

Figure 9. Comparison of HPLC profiles at 280 nm of constitutive phloem extracts (100% methanol) of two Chinese Ash groups: treated and control groups overlay. For peak assignments of the major peaks, see Tables 2 and 3

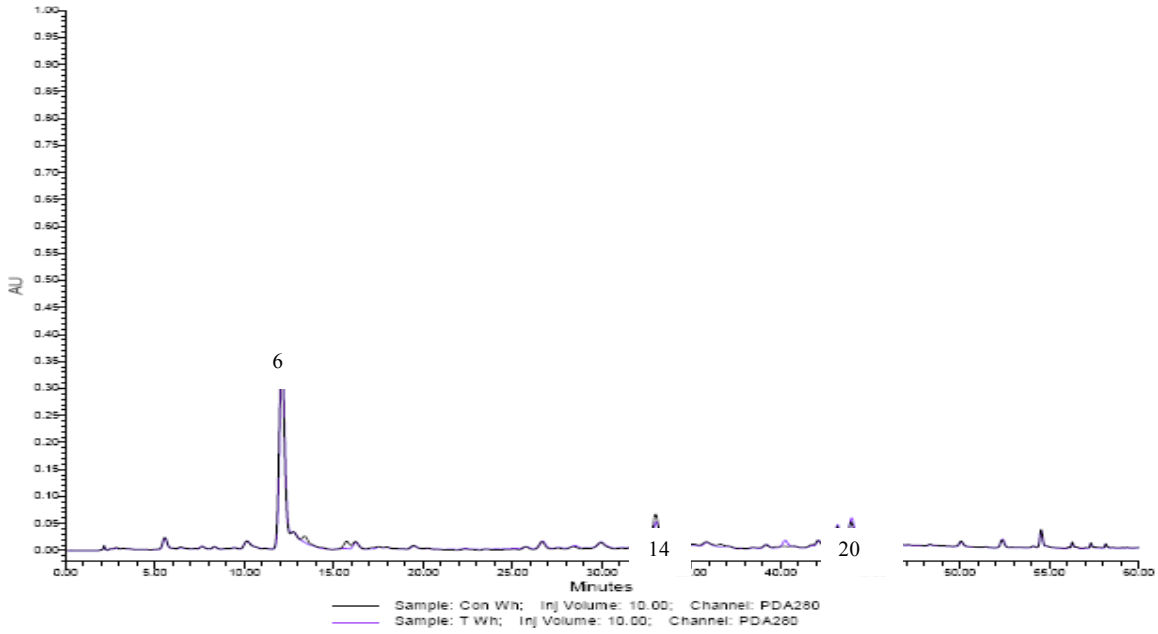

Figure 10. Comparison of HPLC profiles at 280 nm of constitutive phloem extracts (100% methanol) of two White Ash groups: treated and control groups overlay. For peak assignments of the major peaks, see Tables 2 and 3

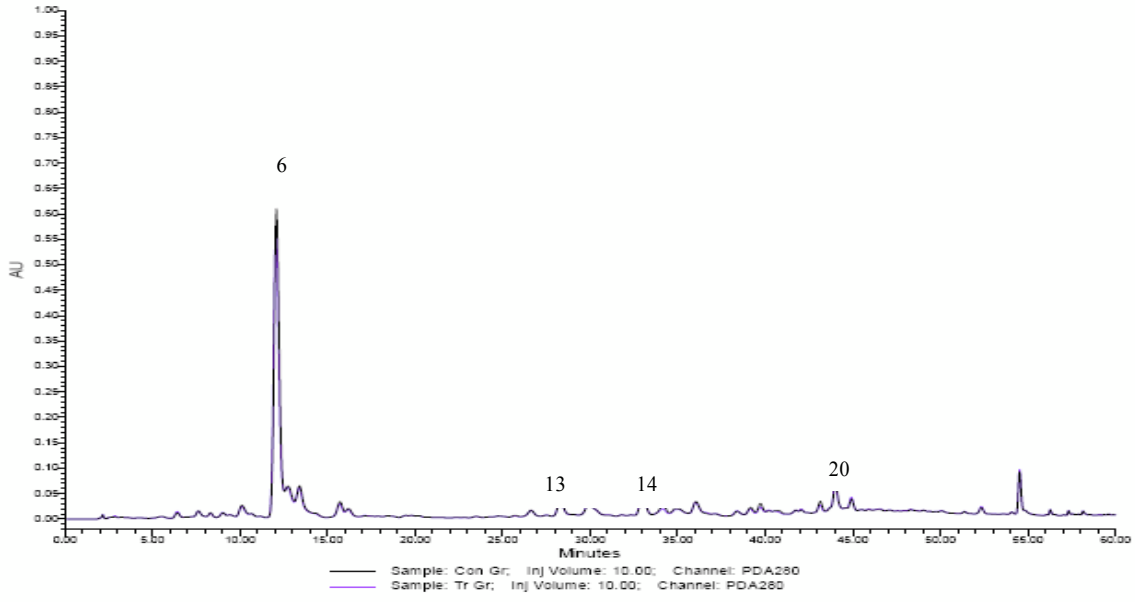

Figure 11. Graph 8. Comparison of HPLC profiles at 280 nm of constitutive phloem extracts (100% methanol) of two Green Ash groups: treated and control groups overlay. For peak assignments of the major peaks, see Tables 2 and 3

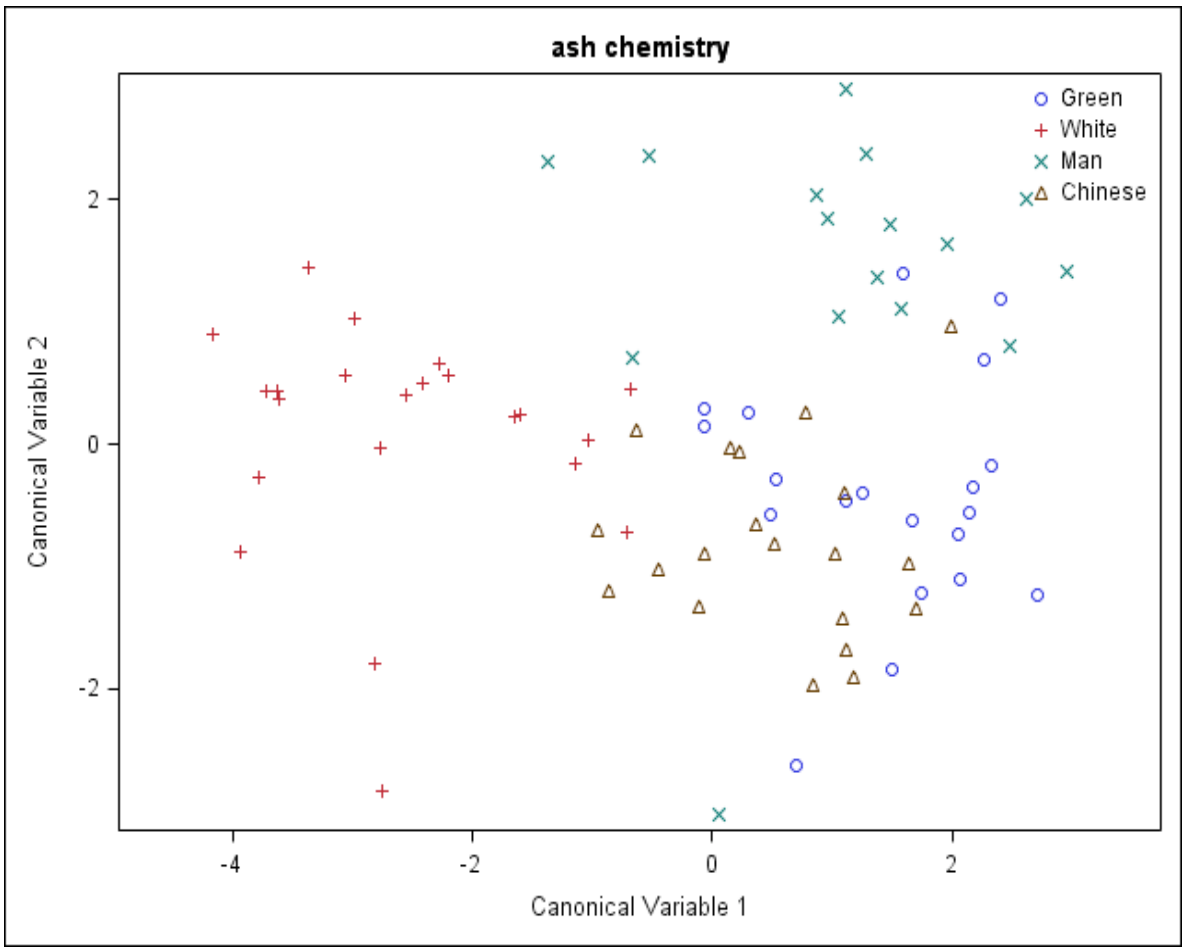

Figure 12. Result of the discriminant Function Analysis of five common compounds (Tyrosol Hexoside, Mandshurin, Pinoresinol compound, Syringaresinol and Oleuropein) all four ash species share.

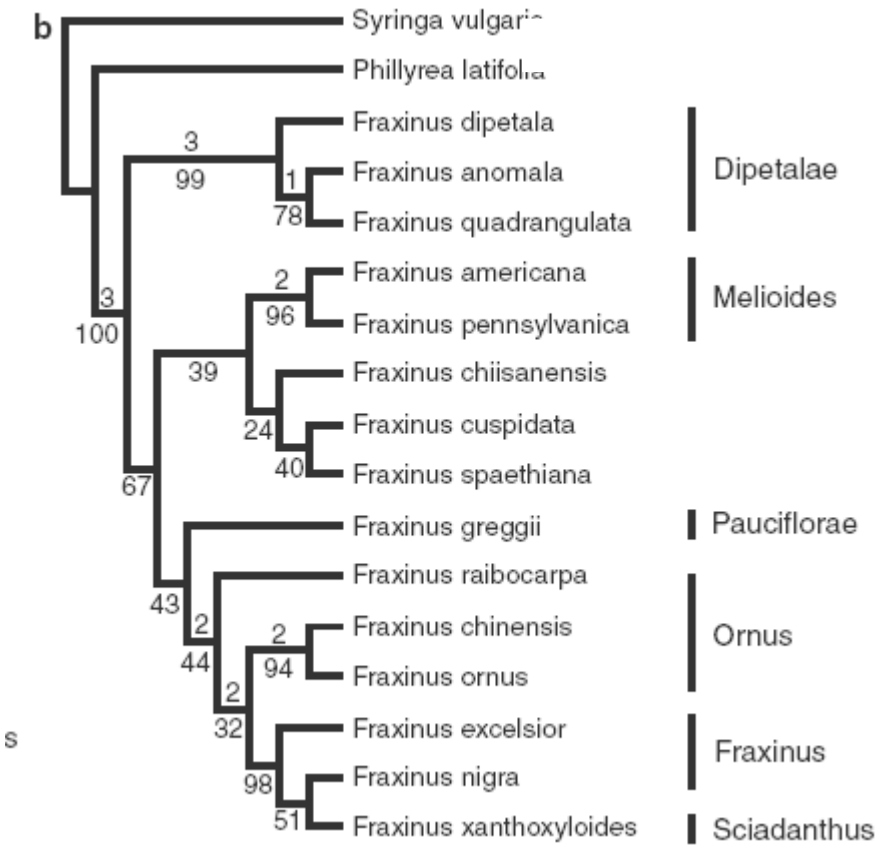

Figure 13. Maximum likelihood tree of a reduced set of the ITS data. Bootstrap values are shown below the branches Wallander E / Plant Syst Evol 273 (2008):25–49