Biochemistry, physiology, and ecology of paper birch defenses to bronze birch borer and their responses to anthropogenic greenhouse gases

Dissertation

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Abstract

Periodic outbreaks by bronze birch borer (*Agrilus anxius*) have caused widespread birch (*Betula*) mortality in the boreal forests of North America. Mechanisms by which deciduous trees resist wood-borers are not understood but have been hypothesized to result from integrated physical and biochemical phloem defenses. Historically, wood-borer outbreaks have been associated with environmental stress, which is thought to weaken tree defenses. Therefore, the overarching goals of this dissertation research were to characterize birch defenses to bronze birch borer, their relationship to whole plant physiology, and how their expression is affected by environmental stress.

Objectives of this research were to use a metabolomics approach to compare the constitutive (*Chapter 2*) and induced (*Chapter 3*) biochemical profiles of paper birch (*B. papyrifera*) phloem, which is resistant to bronze birch borer by nature of its coevolutionary history, with that of European white birch (*B. pendula*), which is much more susceptible. Then, to investigate the relationship of these putative defenses to whole plant physiology, paper birch stems were girdled to disrupt transport of current photosynthate and alter within-tree resistance to bronze birch borer (*Chapter 4*). Finally, to determine how environmental factors modify expression of resistance, the effects of elevated CO$_2$ and ozone (O$_3$) on paper birch resistance and bronze birch borer colonization were analyzed (*Chapter 5*).

Rate of wound periderm (callus) formation did not differ between paper birch and European white birch, but may contribute to resistance if it interacts with defensive
chemicals or nutritional factors that slow feeding, allowing wound periderm to encapsulate larvae. Constitutive and induced phenolic profiles of paper birch and European white birch differed. Paper birch had two phenolics that were not detected in European white birch, and concentrations of six were higher in paper birch than European white birch, which may contribute to higher resistance of paper birch to bronze birch borer.

Trunk girdling decreased phloem resistance to bronze birch borer below the girdle, confirming that stress compromises resistance and supporting the hypothesis that the flux of current photosynthate from leaves to trunk provides the energy to drive defensive responses. Total phenolic concentrations and rate of wound periderm formation were higher above the girdle, corresponding with higher resistance in that portion of the tree.

Elevated atmospheric CO\(_2\) and O\(_3\) decreased paper birch resistance to bronze birch borer in a four-year study at the Aspen FACE facility in Rhinelander, WI. In combination, however, elevated CO\(_2\) and O\(_3\) had no effect on paper birch resistance, which is consistent with previous studies showing elevated CO\(_2\) to ameliorate effects of elevated O\(_3\) on tree physiology. Elevated CO\(_2\) and O\(_3\) altered phloem chemistry but not in ways that could explain patterns of bronze birch borer colonization. The results suggest that on-going changes in atmospheric composition could facilitate bronze birch borer outbreaks, possibly altering the distribution of paper birch in North America.
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Chapter 1

Literature review

Introduction

Wood-boring insects mediate many processes in forest ecosystems including nutrient cycling (Cobb et al. 2010), pollination (Maeto et al. 2002), and releasing understory plants (Gandhi and Herms 2010). A large proportion of buprestids and cerambycids are saproxylic, meaning they are dependent on dead or dying trees for development (Speight 1989, Grove 2002). However, a few species colonize and can kill healthy or weakened trees as they feed in phloem and/or xylem tissues (Anderson 1944, Barter 1957, Cote and Allen 1980, Morewood et al. 2003, Poland and McCullough 2006, Rebek et al. 2008). Outbreaks of these species have been implicated as the major causal agents of region-wide tree mortality, which can have long-term impacts on communities and ecosystems (Balch and Prebble 1940, Stephen et al. 2001, Poland and McCullough 2006, Hu et al. 2009). Widespread tree mortality reduces leaf area, which results in reduced atmospheric carbon dioxide (CO₂) uptake and could cause systems to temporarily be a source of CO₂ (Kurz et al. 2008). Stand mortality also releases understory plants, which can alter successional trajectories and alter nutrient cycling due
to the large influx of coarse woody debris to the forest floor (Bouchard et al. 2006, Brassard and Chen 2006, Duchesne and Ouimet 2008, Gandhi and Herms 2010).

Bronze birch borer (*Agrilus anixius* Gory) (Coleoptera: Buprestidae) is a wood-boring beetle indigenous to North America that has been implicated in periodic, widespread birch mortality throughout the 20th century (Balch and Prebble 1940, Barter 1957, Jones et al. 1993b). It is also a pest of ornamental birches throughout North America (Kozel and Toth 1975, Kozel and Smith 1976, Ball and Simmons 1980, Dirr 1981, Santamour 1982, Miller et al. 1991, Dirr 1998). Larvae feed in phloem tissues under the bark producing galleries that disrupt transport of photosynthate. These galleries can girdle a tree, resulting in tree death (Barter 1957).

Although bronze birch borer infests most species of birch, there is substantial interspecific variation in resistance to this insect (Ball and Simmons 1980, Dirr 1981, Miller et al. 1991, Nielsen et al. *in press*). North American species are resistant (Nielsen et al. *in press*), although increased susceptibility of most North American species has been associated with stress (Balch and Prebble 1940, Anderson 1944, Barter 1957, Jones et al. 1993b), whereas European and Asian species are susceptible (Ball and Simmons 1980, Miller et al. 1991, Nielsen et al. *in press*). The mechanisms underlying this variation in resistance among species are not understood. Therefore, the overarching goal of this dissertation research was to analyze potential mechanisms underlying resistance of paper birch (*B. papyrifera* Marshall), a North American species, to bronze birch borer. These potential resistance mechanisms were examined across levels of biological organization, connecting biochemical and physiological mechanisms of resistance with insect population dynamics and climate change.
Bronze birch borer outbreaks

Birch decline

Bronze birch borer outbreaks have been historically associated with stressed birch trees (Balch and Prebble 1940, Anderson 1944, Barter 1957). For example, 100 million paper birch died from bronze birch borer colonization in the Great Lakes region from 1988-89, and tree susceptibility was anecdotally associated with drought stress and advanced age (Jones et al. 1993b). This is consistent with the label “secondary pest,” meaning that bronze birch borer can only colonize and kill trees that have been weakened by stress (Anderson 1944).

The association between birch and bronze birch borer fits into the decline model outlined by Sinclair (1964) and expanded by Manion (1981). This model identified three successive and interactive factors that together result in tree mortality: (1) predisposing factors (e.g. tree age, soil conditions, tree genetics, air pollution) that are general, long-term stresses; (2) inciting factors (e.g. drought, defoliation) that are specific, short-term stresses; and (3) contributing factors (e.g. root rot, cankers, insect borers) that are opportunistic stresses attacking weakened trees. Manion (1981) arranged these categories visually into a disease spiral. In this model, decline events will minimally involve one factor from each category. Predisposing factors initiate the spiral of decline, and inciting and contributing further stress trees ultimately leading to tree death.
Additionally, some birch decline events could also fit into the theory of cohort senescence (Mueller-Dombois 1986, Mueller-Dombois 1992). Cohort senescence occurs within a community of similarly aged trees. Proceeding a period of vigor or growth, stands enter a stage of maturity. Mature plants exhibit decreased energy as a result of intrinsic genetic factors, which when coupled with extrinsic environmental stresses, can lead to large-scale dieback and mortality (Mueller-Dombois 1986). In other words, stand initiation and mortality are a byproduct of a shared life history. For example, major disturbances such as fires, clear-cutting, tornadoes, or hurricanes can result in homogenous stands of trees dominating large areas of forest or can result in mosaic-like cohorts across the forest. These trees, sharing a similar physiological experience, mature together to a senescing life stage and an abiotic or biotic factor (e.g. injury by wood-boring beetle) triggers their decline. The trigger itself is not the sole causal agent of dieback but rather a secondary or tertiary factor that occurs in synchrony with the intrinsic nature of the stand/cohort (Mueller-Dombois 1986). Under this model, observed stand mortality is not viewed as a disease, but a normal process of population dynamics and forest ecology (Mueller-Dombois 1992).

*Factors regulating insect herbivore populations*

A contentious debate exists whether top-down (e.g. natural enemies) and/or bottom-up (e.g. resource limitation) factors regulate insect herbivore population dynamics (Hairston et al. 1960, White 1978, Hunter and Price 1992, Power 1992). The top-down view was introduced by Hairston et al. (1960) in their “world is green”
hypothesis. In this hypothesis they argue that predators limit the abundance of herbivores, allowing plants to accumulate. Alternatively, the bottom-up view, suggests that each trophic level is largely food limited (White 1978) and that not all green food is edible because of defenses (Feeny 1968, Coley et al. 1985). Others offer that bottom-up and top-down factors simultaneously regulate populations (Oksanen et al. 1981, Leibold 1989, Power 1992) and that variation in abiotic attributes of the environment as well as community/ecosystem biotic diversity influence the relative strength of these forces (Hunter and Price 1992).

Bronze birch borer populations seem to be regulated by both top-down and bottom-up forces. In studies in Maine and New Brunswick, parasitism of bronze birch borer eggs averaged over 50% (Nash et al. 1951, Barter 1957). However, in Pennsylvania, egg parasitism was only 7%, but larval parasitism was 18% and woodpecker predation of overwintering larvae was 60% (Loerch and Cameron 1983). Woodpecker predation was also recorded to cause 51% of larval mortality in individual trees in New Brunswick (Barter 1957). This level of egg and larval mortality due to natural enemies could at least partially regulate populations.

It is clear that bottom-up forces also partially regulate bronze birch borer populations as evidenced by differential survival of larvae among resistant and susceptible host species (Ball and Simmons 1980, Miller et al. 1991, Nielsen et al. in press). Many larvae that colonize resistant birch species never survive to adults and die in the tree (Anderson 1944, Barter 1957, Nielsen et al. in press). Moreover, outbreaks of bronze birch borer have long been associated with stressed trees, implying that host
resistance has been compromised, allowing increased survival of bronze birch borer
(Balch and Prebble 1940, Anderson 1944, Barter 1957, Jones et al. 1993b).

**Introduction to plant defense and resistance**

The field of plant-insect interactions emerged, at least in part, from the desire to understand the *raison d'être* for the abundant diversity of secondary metabolites found in plants (Dethier 1954, Fraenkel 1959). These metabolites were first thought to have no function and were considered “waste products” of primary metabolism that accumulated in plants due to the lack of an excretory system. However, another view began to emerge that secondary metabolites provide an ecological function as a plant defense against herbivores and pathogens (Dethier 1954, Fraenkel 1959, Ehrlich and Raven 1964).

Plants limit the impact of herbivores via three modes: antixenosis, antibiosis, and tolerance (Painter 1958, Beck 1965, Painter 1965, Hanover 1975, Larsson 2002). Antixenosis describes plant traits that negatively affect the behavior of an insect. For example, the absence of a trait could disable recognition of a host or the presence of a trait could repel an insect from a host (Kogan and Ortman 1978). Antibiosis describes plant traits that negatively affect the physiology of an insect. Collectively, antixenosis and antibiosis describe possible mechanisms of plant resistance as they negatively affect insect behavior or physiology. Tolerance, on the other hand, does not detrimentally affect insect performance. Rather it describes an alternative plant defense strategy to cope with insect herbivory: the ability to regrow and/or reproduce after herbivory (Painter 1958, Vandermeerijden et al. 1988, Strauss and Agrawal 1999). Much progress has been made in
understanding the mechanisms of resistance of herbaceous and woody plants to folivores and gymnosperms to bark beetles (e.g. Beck 1965, Raffa and Berryman 1983, Raffa 1991, Haukioja 2003, Howe and Jander 2008, Agrawal and Konno 2009). However, the mechanisms mediating interactions between woody angiosperms and wood-boring insects are poorly understood.

Plant resistance mechanisms negatively impact insect behavior or physiology (Painter 1958, 1965, 1968, Larsson 2002). Mechanisms affecting insect physiology, specifically, can reduce growth, fecundity and/or survival (Larsson 2002). Many plant traits, both nutritive and defensive, are known to impact insect performance. Therefore, it could be predicted that in some cases, multiple traits contribute to the relative resistance of a species or plant because many dimensions determine plant quality.

There is a veritable plethora of plant traits that could contribute to resistance of a plant to an insect. Moreover, the expressed mechanisms could vary by genotype, evolutionary history, phylogeny, tissue, environment, and herbivore(s) present. Physical mechanisms of plant resistance include leaf trichomes (Loe et al. 2007, Kaplan et al. 2009, Holeski et al. 2010), leaf toughness (Matsuki and Maclean 1994, Peeters et al. 2007, Kawasaki et al. 2009), wound periderms (Anderson 1944, Dunn et al. 1990), and high sap flow (Raffa 1991, Morewood et al. 2004). Biochemical mechanisms of resistance include both defensive components like terpenes, phenolics, tannins, lignin, and nutritive components like proteins, sugars, amino acids, and sterols (reviewed below). Additionally, many of these traits can be constitutively expressed or can be induced in response to insect feeding (Green and Ryan 1972).
Before further discussion, it is noteworthy that the dichotomous classification that secondary metabolites are defenses and primary metabolites are nutrients is a useful generalization but is frequently false (Berenbaum 1995). In fact, the dichotomy that a compound is either a defense or a nutrient can also fail. These definitions or categorizations are dependent upon context, not biosynthetic origin (Janzen 1979, Reese 1979, Berenbaum 1995). Not all secondary metabolites serve a defensive function against a particular herbivore or perhaps any herbivore. Secondary metabolites can have a neutral or even positive affect on herbivore performance (Bernays and Woodhead 1982, Raubenheimer and Simpson 1990). For example, the tree locust (*Anacridium melanorhodon* (Walker)) exhibits increased performance and survival when feeding on lettuce with phenol supplements, possibly because phenols may stabilize proteins in the locust cuticle (Bernays and Woodhead 1982). The ecological role of primary metabolites or nutrients is equally situation dependent. Nutrients can be harmful to herbivores when concentrations are too high, too low, or when imbalanced (Slansky 1992, Simpson and Raubenheimer 2001, Lokvam et al. 2006). However, unless otherwise noted, these traditional classifications will be implied.

*Wound periderm*

Chewing by insects causes substantial physical damage to plant tissues. Injury from feeding not only results in tissue loss but also increases vulnerability to invasion by pathogens and microbes and increases fluid loss. Plants respond to physical injury,
including injury produced by chewing insects, by production wound periderms (i.e. callus tissue) (Ginzberg 2008).

After physical injury, plants cells dieback at the wound surface. Just underneath these dead cells an impervious tissue forms, which is a few cells thick and contains deposits of suberin and/or lignin (Biggs 1985). These cells minimize further damage by isolating wounded tissue from unwounded, healthy tissue and by serving as a temporary barrier to water loss and microbial invasion until the permanent wound periderm forms (Walter and Schadel 1983, Biggs 1985, Ginzberg 2008). Wound periderms originate from the phellogen (cork cambium) or from individual cells that act as mother cells. These meristematic cells then differentiate and reestablish phloem and cambial integrity (Biggs 1985).

The rate of wound periderm formation over exposed tissue, especially suberization of the outer phellem cells, is fundamental to host resistance against pathogens (Kolattukudy 1984, Biggs 1992, Simard et al. 2001, Aoun et al. 2009). However, wound periderm formation may also be an important component in resistance of woody angiosperms to wood-boring insects (Dunn et al. 1990). Wound periderms are thought to have a passive defense function against insects (Biggs et al. 1984). It has been hypothesized that constitutive and/or induced secondary metabolites (e.g. phenolics, tannins, lignin) may inhibit larval growth (and thus velocity of phloem excavation) to the point that the larvae may be encapsulated by wound periderm tissue induced by feeding injury (Eyles et al. 2007).
Low-molecular weight phenolics

Phenolics are ubiquitous in woody angiosperms (Julkunen-Tiitto et al. 1996, Ossipov et al. 1996, Osier et al. 2000, Salminen et al. 2004, Barbehenn et al. 2005, Eyles et al. 2007, Pereira et al. 2007, Orians et al. 2010). Phenolics are molecules that contain at least one phenol, which structurally is a hydroxylated benzene ring. Flavonoids, tannins, and lignin are all classes of phenolics but vary in structure and molecular weight. All of these compounds are derived from the shikimate pathway (Knaggs 2003).

Low-molecular weight phenolics, like flavonoids, have been extensively studied and include thousands of compounds including anthocyanidins, catechins, flavonols, flavones, and isoflavonoids (Harborne 1991). Biological functions of phenolics are highly variable and include UV protection, signaling molecules, pigments to attract pollinators, and defenses against microbes, fungi, and herbivores (Harborne 2001, Buer et al. 2010).

Many simple phenolics have been ascribed a defensive function against herbivores, working as antifeedants, digestibility reducers, or toxins. In *Eucalyptus*, two phloroglucinols, macrocarpal G and jensenone, are strong antifeedants to possum and koala (Lawler et al. 1998). Platyphyllloside, a diarylheptanoid common in *Betula*, decreases ruminant digestion of nutrients (Sunnerheim et al. 1988, Sunnerheim and Bratt 2004). The flavonoid fraction of *Elodea nuttallii* Planch. (Hydrocharitaceae), an invasive macrophyte, deters larval feeding and reduces growth of the generalist aquatic moth, *Acentria ephemerella* (Pyralidae) (Erhard et al. 2007). Angiosperms belonging to Anacardiaceae produce toxic phenols, including alkylcatechols, alkylreorcinols and
biflavonoids, that can restrict growth of pathogenic fungi like *Alternaria* (Cojocaru et al. 1986) and are thought to be toxic to insects and vertebrates (Joel 1980, Mitchell 1990, Farrell et al. 1991).

Some insect species that are adapted or that share a coevolutionary history with their hosts can sequester phenolics for their benefit. For example, some butterfly larvae (e.g. *Polyommatus icarus* Rottemburg) sequester UV-absorbing flavonoids from their hosts. These flavonoids are then expressed in the adult wing coloration, which are used to attract mates or deter predators (Burghardt et al. 2000, Burghardt et al. 2001, Knuttel and Fiedler 2001, Ferreres et al. 2007).

*Tannins*

Tannins are polyphenolics with high molecular mass. They exhibit the characteristics of being water soluble and able to precipitate proteins (Hagerman and Butler 1991, Ayres et al. 1997). Tannins are further classified into hydrolysable and condensed tannins. Hydrolyzable tannins are esters of sugars with gallic acid whereas condensed tannins are polymers of flavonols.

Tannins have long been implicated in mediating interactions between insects and plants (Feeny 1976, Schultz and Baldwin 1982, Rossiter et al. 1988). Negative associations have been observed between concentrations of induced tannins and insect performance (Schultz and Baldwin 1982, Rossiter et al. 1988, Ruuhola et al. 2008), and between phenological changes in concentrations of tannins and insect performance (Feeny 1970).
Tannins are typically measured by crude extracts of “total phenolics,” which includes tannins or “total tannins” using colorometric analysis. It is becoming increasingly apparent that these crude measures fail to capture the biological role and varying effects that a suite of tannins can have on insect physiology (Moilanen and Salminen 2008). It is predicted that individual tannins, which are variable in structure, will have different biological activities and impact on herbivore species (Feeny 1976, Ayres et al. 1997, Salminen et al. 2004).

Tannins were first thought to act quantitatively, impeding protein digestion by insect herbivores. Specifically, tannins were thought to bind proteins and enzymes in the insect gut, reducing nutritional quality (Feeny 1976). However, many studies fail to indicate that tannins affect protein assimilation (Bernays et al. 1980, Bernays and Chamberlain 1980, Martin et al. 1987).

Alternatively, recent evidence indicates that ingested tannins impact insect physiology via prooxidant activity (Martin et al. 1987, Appel 1993, Summers and Felton 1994). The prooxidant activity of tannins is particularly apparent in high pH environments like those found in caterpillar midguts (Appel 1993, Barbehenn and Martin 1994). According to this prooxidant view, when tannins in a gut are oxidized they produce reactive oxygen species (e.g. semiquinones, quinones, hydroxyl radicals, and peroxides). Reactive oxygen species then damage nutrients and/or produce oxidative stress in the midgut (Bi and Felton 1995, Barbehenn et al. 2005).

There is variation in prooxidant activity of hydrolysable versus condensed tannins. Hydrolyzable tannins (i.e. ellagitannins, galloyl glycosides) are highly reactive whereas condensed tannins are relatively unreactive (Barbehenn et al. 2006a, Moilanen...
and Salminen 2008). In fact, many recent studies suggest that condensed tannins have minimal impact on herbivore performance (Rossiter et al. 1988, Ayres et al. 1997, Kopper et al. 2002). For example, whitemarked tussock moth (*Orgyia leucostigma* (J. E. Smith)) feeding on diets high in condensed tannins exhibit protracted developmental time, but survivorship is unaffected (Kopper et al. 2002). Within hydrolysable tannins, ellagitannins are more prone to oxidize than galloyl glycosides (Barbehenn et al. 2006).

**Lignin**

Lignins are polymers of highly methoxylated phenolics produced by the phenylpropanoid pathway. Due to the complexity and variability of lignin structures, synthesis was thought to be a random process. Recent evidence suggests that there is organization among monomers within each polymer, but monomer composition varies by ontogeny, cell type and species (Whetten and Sederoff 1995, Boerjan et al. 2003).

Lignin is present in all vascular plants, although lignin content of herbaceous plants is comparatively depauperate to that of woody plants (Sarkanen and Ludwig 1971). The primary function of lignin is structural as it helps to maintain the strength, rigidity, and water impermeability of the vascular system (Whetten and Sederoff 1995). However, lignin is also thought to serve a defensive function against herbivores, microbes, and pathogens and can reduce the digestibility of cell wall carbohydrates like cellulose and hemicellulose or physically deter/inhibit insect and pathogen colonization (Vance et al. 1980, Hammerschmidt and Kuc 1982, Rangasamy et al. 2009).
The effectiveness of lignin as a defense to herbivores is variable and somewhat inconclusive. Many studies showed no association between lignin and herbivore performance. For example, feeding preference and performance of both geometrids and chrysomelids were not associated with lignin concentrations in transgenic versus control European white birch (Tiimonen et al. 2005). Similarly, reduced lignin content of transgenic aspen (*Populus tremuloides* Michaux) was not linked to reduced feeding and/or performance of gypsy moth larvae (*Lymantria dispar* L.) or forest tent caterpillars (*Malacosoma disstria* Hübner) (Brodeur-Campbell et al. 2006). In these studies, however, the exact role of lignin to herbivore performance may have been confounded or camouflaged by potential pleitrophic effects of transgenes (James et al. 1998).

Other studies have suggested an inverse, dose-dependent relationship between lignin concentrations and insect performance. For example, increased lignin concentrations of Norway spruce (*Picea abies* (L.) Karst) and American Sitka spruce (*P. sitchensis* (Bong.) Carr.) reduced survival, growth, and weight of *Dendroctonus micans* (Kugelann) larvae (Wainhouse et al. 1990). Additionally, sorghum varieties resistant to sorghum shoot fly (*Atherigona varia soccata* Rond.) are characterized by high lignification of cells walls and greater density of silica bodies. These traits of sorghum are thought to physically prevent penetration by neonates (Blum 1968). Additionally, lignin content in grasses is associated with reduced digestibility (via reduced enzymatic degradation) of cell wall carbohydrates in ruminants (Chesson 1988, Besle et al. 1994).
**Nitrogen, amino acids, and proteins**

Numerous studies have demonstrated that nutritive components of plants can influence host selection and foraging by insects as well as growth and performance. Plant nitrogen content (and the quality thereof) is considered to be particularly important to insect herbivores (McNeill and Southwood 1978, Mattson 1980, Brodbeck and Strong 1987, White 1993). Both the quantity and form of nitrogen in plants are significantly different from that of herbivores, and these differences are considered major nutritional barriers in utilization of plant food (Hinton 1977, Berenbaum 1995). Southwood (1973) suggested that insect growth should be nitrogen limited because, for example, leaf tissue is 2% nitrogen whereas insect tissue is 30-40% nitrogen.

Because nitrogen is thought to be an important nutritional factor limiting insect herbivores, total N content is frequently used as a proxy of nutritional quality of plants. However, it is unlikely that herbivores respond to total pools of nitrogen because their physiological needs are multivariate and dynamic (Brodbeck and Strong 1987, Berenbaum 1995, Simpson and Raubenheimer 2001). Rather, an increase in the specific nutrient(s) most limiting insect growth would provide the greatest benefit to insect performance (i.e. Liebig’s law of minimum). Additionally, the form of nitrogen determines the efficiency in which the insect can utilize the compound. For example, free amino acids can be readily metabolized whereas complex proteins require catabolic processing before utilization (Cockfield 1988).
There are 10 essential amino acids that insects cannot synthesize and must obtain from their host or from gut endosymbionts. These are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (House 1962). However, the majority of amino acids in plants are non-essential amino acids, which vary in concentration by ontogeny, season and stress (Brodbeck and Strong 1987, Bi et al. 2003, Bi et al. 2007, Gould et al. 2007). The presence of endosymbionts can enable insect species to utilize hosts that would otherwise be nutritionally inadequate. For example, the endosymbiont *Buchnera* supplies aphids with tryptophan, which aphids require but is extremely low or not detectable in phloem sap of their hosts (Douglas and Prosser 1992, Lai et al. 1994).

Low concentrations of plant nutrients may lead to compensatory feeding by insects (Slansky 1993, Kause et al. 1999). Although compensatory feeding increases the possibility of consuming limiting nutrients, it also increases the possibility of consuming defensive compounds (Broadway and Duffey 1988, Slansky and Wheeler 1992). The concomitant increase in consumption of allelochemicals could be deleterious to insect performance and survival.

Many plant proteins have also been implicated as plant defenses (Carlini and Grossi-de-Sa 2002). Proteinase inhibitors have been extensively studied and inhibit insect proteases from catabolizing plant proteins in the insect gut (Green and Ryan 1972, Jongsma and Bolter 1997, Fan and Wu 2005). However, many insects express proteases that are insensitive to these inhibitors or that can inactivate the plant’s protease inhibitors (Jongsma and Bolter 1997). Polyphenol oxidases, which are enzymes that oxidize
phenolics to quinones, can increase oxidative stress and have also been implicated as defense mechanisms (Felton et al. 1992, Bhonwong et al. 2009).

*Other nutritive compounds*

Sterols have also been identified as an important nutrient mediating plant-herbivore interactions (Behmer and Nes 2003). Sterols are triterpenoid steroid alcohols that are incorporated into cell membranes to maintain integrity, permeability, and fluidity (Svoboda 1999). They are also precursors for steroid hormones that regulate gene expression (Behmer and Nes 2003).

In most insects, cholesterol is the primary sterol utilized in both cell membranes and for synthesis of steroid hormones (e.g. ecdysteroids). Insects cannot synthesize cholesterol *de novo* but metabolize phytosterols into cholesterol (Clark and Bloch 1959, Feldlaufer and Svoboda 1989). The most common phytosterols are sitosterol, stigmasterol, and spinasterol. Phytosterols are structurally very similar to cholesterol, but have ethyl- or methyl groups at carbon position 24 and/or double bonds at positions other than five in the tetracyclic nucleus (Janson et al. 2009).

*The multivariate and dynamic nature of plant defense*

It is frequently difficult to predict how well defended a plant will be against a specific herbivore. In some cases, this difficulty may arise from the complexity of traits that underlie the nature of defense. It is evident that both primary and secondary
metabolites of plants can simultaneously influence insect growth and survival (Slansky 1992, 1993, Haukioja 2003, Howe and Jander 2008). However, research and theory in the field of plant-insect interactions frequently focuses on the role of plant secondary chemicals (i.e. defensive compounds) or primary chemicals (i.e. nutritive compounds) on insect performance. Additionally, plant defense theories are phytocentric (Glynn et al. 2003, Mattson et al. 2005). They make predictions about how well-defended a plant will be based on genotypic variation (e.g. growth rate hypothesis (Coley et al. 1985)), environmental variation (e.g. carbon:nutrient balance hypothesis (Bryant et al. 1983), growth differentiation balance hypothesis (Loomis 1953, Herms and Mattson 1992)) or both (e.g. optimal defense hypothesis (Feeny 1976, Rhoades and Cates 1976)). In other words, they focus on the factors modulating allocation of carbon to secondary metabolites in the plant but do not predict if the plant will be more or less resistant to an herbivore. Increased allocation to the pool of secondary metabolites or a particular metabolite may not necessarily result in the plant being more defended against an insect (Agrawal and Fishbein 2006). The insect may be an adapted specialist to a plant and its secondary metabolites, which under some contexts would render the “defenses” ineffective (Wittstock et al. 2004), or the threshold necessary to elicit a negative behavioral or physiological response may not be met. Haukioja (2003) suggests that defense (defined as chemical, physical, and ecological plant mechanisms that reduce herbivore damage) can only be understood when relating both primary and secondary metabolites to insect consumption and performance.

Plants experiencing natural conditions express variable ratios of both individual and pools of primary:secondary metabolites in both time and space (Kause et al. 1999).
This is because allocation and accumulation of particular metabolites varies by ontogeny, environment, genotype, and interactions between the environment and genotype (Feeny 1976, Mattson 1980, Bryant et al. 1983, Coley et al. 1985, Herms and Mattson 1992, Osier and Lindroth 2001). Therefore, the expectation for consistent negative correlations between insect performance and concentrations of putatively defensive compounds could be unrealistic. Other phytochemicals, like essential nutrients and water, which also contribute to insect performance, are in flux (Haukioja 2003). This fluctuation can influence the effectiveness of defensive compounds and in themselves have consequences on insect fitness (Slansky 1992, Haukioja 2003, Zangerl and Berenbaum 2004).

One experimental approach to understand how the multidimensional biochemical composition of food affects animal physiology is ecological stoichiometry. Experiments utilizing the ecological stoichiometry approach indicate that increasing nutrients can both increase and decrease the deleterious effects of allelochemicals. In other words, the efficacy of an allelochemical as a defense can be dependent on the nutritional background (Simpson and Raubenheimer 2001). Summarizing research findings from this experimental approach, Simpson and Raubenheimer (2001) outline three factors that determine food quality: (1) the organism’s current nutritional state in respect to its multiple nutrient needs; (2) the composition and quantity of nutrients present in food; and (3) the quantity and ratios of nonnutritive and/or defensive compounds.

Many studies indicate that plant defense to a particular herbivore involves more than one plant trait. For example, sugar maple is thought to be relatively resistant to *L. dispar* because of high levels of hydrolysable tannins and low levels of ascorbate and proteins. Together these factors increase oxidative stress in caterpillars and reduce
nutritional value, which decreases performance (Barbehenn et al. 2009). Soybean is thought to be resistant to *Helicoverpa zea* (Boddie) due to increases in phenolics and decreases in antioxidants and proteins (Bi and Felton 1995). In tobacco, nicotine and protease inhibitors work synergistically, conferring resistance against Lepidoptera (Steppuhn and Baldwin 2007).

**Resistance mechanisms of woody angiosperms to wood-boring beetles**

The physical and biochemical mechanisms of resistance employed by gymnosperms against bark beetles have been extensively studied. In brief, gymnosperm defense consists of two important components: (1) a preformed resin system that can pitch out colonizing bark beetles and deter colonization and (2) a hypersensitive response that is characterized by both the induction of terpenes and necrotic lesions that can confine the beetle (Raffa and Berryman 1983, Raffa 1991). Comparatively, very little is known about the resistance mechanisms employed by woody angiosperms against wood-boring beetles (Coleoptera: Buprestidae and Cerambycidae). However, with the introduction of exotic wood-boring beetles threatening the survival of North American tree species, the gap in knowledge in this field has become an academic and public priority.

There have been a limited number of studies aimed at understanding the resistance of angiosperms, and a few physical and chemical mechanisms have been proposed. Golden rain trees (*Koelreuteria paniculata* Laxmann) physically expel Asian longhorned beetles (*Anoplophora glabripennis* (Motschulsky)) from stems via high sap
flow (Morewood et al. 2004). High water content in phloem tissues of *Eucalyptus* trees physically prevents the eucalyptus longhorned borer (*Phoracantha semipunctata* (Fabricius)) from reaching the cambium (Hanks et al. 1991, Hanks et al. 1999). The rate of wound periderm formation in *Quercus* and *Betula* is hypothesized to function defensively against red oak borer (*Enaphalodes rufulus* (Haldeman)), two-lined chestnut borer (*Agrilus bilineatus* (Weber)) and bronze birch borer by encapsulating feeding larvae (Anderson 1944, Dunn et al. 1990, Fierke and Stephen 2008). The resistance of callery pear (*Pyrus calleryana* Decaisne) to the Asian longhorned beetle is thought to operate through the presence of uncharacterized chemicals in the bark or phloem tissues (Morewood et al. 2004). Finally, the presence of specific phenolics in Asian ash species like Manchurian ash (*Fraxinus mandshurica* Rupr.) is thought to contribute to resistance to emerald ash borer (*Agrilus planipennis* Fairmaire) (Eyles et al. 2007).

Although bronze birch borer infests most species of birch, there is substantial interspecific variation in resistance to this insect (Ball and Simmons 1980, Dirr 1981, Miller et al. 1991, Nielsen et al. *in press*). North American birch species are resistant to bronze birch borer (Nielsen et al. *in press*), although increased susceptibility has been associated with stressors such as drought (Jones et al. 1993b), high temperatures (Jones et al. 1993b), advanced age (Balch and Prebble 1940), and experimental girdling (Anderson 1944, Barter 1957). In contrast, European and Asian birch species are much more susceptible to bronze birch borer colonization (Ball and Simmons 1980, Miller et al. 1991, Nielsen et al. *in press*). This pattern is consistent with biogeographic theory for plant defense, which predicts that evolution of traits conferring plant resistance to herbivores corresponds with geographic patterns of selection imposed by herbivores.
(Bryant et al. 1994, Swihart et al. 1994). In other words, as herbivory varies along gradients, so will evolution of resistance in hosts, resulting in populations and species that vary in resistance to the coevolved herbivore (Gandhi and Herms 2010). Therefore, North American birch species are hypothesized to be resistant to bronze birch borer because of a coevolutionary history with this insect. In contrast, European and Asian species are more susceptible because they have not been exposed to bronze birch borer, as they are geographically separate (Nielsen et al. in press).

Interspecific variation in birch resistance to bronze birch borer might be explained mechanistically by the hypothesis proposed by Eyles et al. 2007. This hypothesis postulates that woody angiosperm resistance to wood-borers results from integration of physical defenses and specific biochemical defenses that are selected by coevolution with a wood-borer. Specifically, the presence of induced, targeted defenses selected by coevolution may slow the growth and movement of larvae, allowing wound periderm to encapsulate larvae. Support for this hypothesis is illustrated by the correlation between the fast growth rate of wound periderm and resistance to bronze birch borer in paper birch (Miller et al. 1991). However, in more susceptible European white birch (B. pendula Roth), there was no relationship between growth rate of wound periderm and bronze birch borer resistance (Miller et al. 1991). Thus, rate of wound-periderm growth alone does not confer resistance in birch but might contribute to a resistance mechanism when present in conjunction with toxic secondary metabolites that have been selected for in species that share a coevolutionary history a wood-boring insect.

Additionally, previous research has suggested that the absence of the phenolic rhododendrol may underlie birch resistance to bronze birch borer (Santamour 1990,
Santamour and Lundgren 1997). Rhododendrol has been implicated as a short-range stimulus for oviposition by mated bronze birch borer females (Santamour 1990) so species lacking this compound do not trigger oviposition and are thus resistant. Rhododendrol naturally occurs in many birch species and is also generated by the hydrolysis of glycosides containing rhododendrol, like betulside (also known as rhododendrin) and betulside pentoside (Smite et al. 1993, Julkunen-Tiitto et al. 1996, Santamour and Lundgren 1997).

**Source-sink interactions in relation to plant defenses**

At the whole plant level, resource allocation depends on strength of sources (the amount of resources readily available for transport) and strength of sinks (e.g. growing meristems including fruits, leaves and flowers), and the vascular connectivity among sources and sinks (Watson and Casper 1984, Herms and Mattson 1992, Honkanen et al. 1994, Carvalho et al. 2006, McCormick et al. 2006). Carbon is exported from sources, leaves, and storage organs to sinks via the phloem vasculature. Source strength is dependent on the amount of carbon available for translocation and sink strength is dependent on the size and metabolic activity (e.g. Herms and Mattson 1992, Marcelis 1996, McCormick et al. 2006). Vascular architecture can restrict movement of photosynthate and nutrients to certain pathways (Watson and Casper 1984, Honkanen et al. 1994, Arnold et al. 2004).

The carbon needed for synthesis of induced defenses in plants comes from both local starch reserves and long-distance transport of carbohydrates in the phloem.
The defensive capacity of stems of conifers against bark beetles and associated fungi is dependent on the ability to mobilize carbohydrates needed for biosynthesis of induced defenses at the site of damage or infestation (Christiansen and Ericsson 1986, Christiansen et al. 1987). In fact, it has been proposed that resistance of conifers against bark beetles is compromised when the local demand for carbohydrates needed for the production of resins and other defenses is larger than the translocation capacity of the phloem (Christiansen and Ericsson 1986). Sink strength also partly determines the ability to induce defenses (Haukioja 1991, Honkanen et al. 1999). For example, as leaves age and their sink strength decreases, their plasticity and wound-responsiveness decreases (Karban and Baldwin 1997), which can result in a lack of accumulation of phenylpropanoids in response insect injury (Arnold and Schultz 2002).

Distribution of induced defenses in plants is also dependent on vascular connectivity because signaling molecules that induce resistance must travel through the plant (Jones et al. 1993a). These signaling molecules then trigger induced defenses, such as proteinase inhibitors (Orians et al. 2000). When a leaf is damaged by an herbivore, leaves that are orthostichous, meaning vertically arranged, share direct vasculature and exhibit induced resistance. However, leaves that are non-orthostichous do not share connectivity with damaged leaves and do not exhibit induced resistance (Murray et al. 1982, Jones et al. 1993a). Additionally, the flow of photosynthate among sources and orthostichous sinks is positively correlated to phenylpropanoid production and induced resistance (Arnold et al. 2004). These observations indicate that plant response to herbivory is not truly systemic and instead modular (Arnold et al. 2004).
The connectivity of the vascular system can also influence transport of photosynthate and nutrients from shoots to roots and vice versa (Orians and Jones 2001). For instance, defoliation of specific branches can reduce growth and survival of roots that have high vascular connectivity to those branches (Murphy and Watson 1996). The flow of nutrients (e.g. phosphorus) from roots to shoots is also restricted by vascular architecture (Rinne and Langston 1960, Hay and Hamilton 1996).

Girdling is a horticultural and experimental technique that disrupts the connectivity among sources and sinks. In this technique, a section of bark and phloem tissue is removed from a branch or trunk of a tree. This technique stops transport of current photosynthate from the canopy to tree parts below the girdle but has little effect on xylem transport of water, nutrients, and solvents (Noel 1970). The result is that photosynthate accumulates above the girdle, often increasing flowering and yield of fruits (Lahav et al. 1971, Jordan and Habib 1996, Goren et al. 2004). On the other hand, tree parts below the girdle experience a shortage of photosynthate, which eventually results in depletion of carbohydrate reserves and eventual root death (Weaver and McCune 1959, Li et al. 2003).

Girdling alters tree resistance to wood-borers by disrupting source-sink interactions (Anderson 1944, Barter 1957). In girdled birch trees, portions of the trunk below the girdle are more susceptible to bronze birch borer than areas above the girdle (Anderson 1944, Herms 1991). Resistance above the girdle is attributed to more vigorous cambial activity (i.e. increased growth rate of wound periderm) (Anderson 1944), which may be fueled by transport of current photosynthate from leaves. Conversely, tissues below a girdle are isolated from long-distance translocation of carbohydrates from
sources above the girdle, and may exhibit a reduced capacity to induce defenses as local reserves are diminished. Girdling mediates interactions between ash and emerald ash borer by increasing the production of sequiterpenes from bark tissues, which is attractive to emerald ash borer (Crook et al. 2008), and by increasing female preference to feed on leaves of girdled trees (Chen and Poland 2009).

Global climate change

*Effects of elevated CO$_2$ and O$_3$ on plant growth, physiology, and chemistry*

Anthropogenically-generated increases in carbon dioxide (CO$_2$) and ozone (O$_3$) continue to impact forest ecosystems. Concentrations of CO$_2$ are rising at a rate of approximately 2 ppm per year (0.6% per year) (Moore and Braswell 1994) and have reached 386 ppm, which is the highest concentration in the last 26 million years (Pearson and Palmer 2000). Concentrations of CO$_2$ are predicted to reach from between 550 ppm to 900 ppm by the end of the century (Karl et al. 2007). Concentrations of O$_3$ have increased on average by 38% (increasing regionally by 20-50%) since the beginning of the industrial revolution (Denman et al. 2007).

Carbon dioxide is the substrate for photosynthesis, and thus an increase in CO$_2$ alters growth/allocation, physiology, and biochemistry. Typically plants have increased rates of photosynthesis and growth (Saxe et al. 1998), and as stomatal conductance declines, reduced water loss via transpiration when grown in enriched CO$_2$ environments (Long et al. 2004). Plants also typically exhibit a higher C:N ratio (McGuire et al. 1995),
and leaf N content decreases by 16% relative to leaves grown in ambient levels of CO$_2$ (Curtis and Wang 1998). This reduction is largely due to decreases in Rubisco concentrations (Bowes 1991, Tissue et al. 1993) and is often associated with increases in phenolics and carbohydrates (Peñuelas and Estiarte 1998, Lindroth 2010), especially starch (Saxe et al. 1998, Stiling and Cornelissen 2007). Although N concentrations decrease, plants generally have improved N use efficiency. This, coupled with increased rates of photosynthesis and higher water use efficiency, accelerates plant growth and is known as the “fertilizer effect” (Beedlow et al. 2004). However, this effect diminishes over times as N becomes limiting at the stand level (Norby et al. 2010).

Plant defense theory predicts that plants under a C-enriched environment will exhibit an increase in C-based chemical defenses and C:N, proportional to the C surplus generated by photosynthesis relative to demands of growth and storage (Herms and Mattson 1992, Mattson et al. 2005). However, changes in concentrations of secondary metabolites appear to be specific to groups of C-based defenses. Findings indicate that CO$_2$ more strongly impacts the shikimic pathway, which produces phenolics, than the mevalonic acid and methylerythritol phosphate pathway, which produce terpenes (Koricheva et al. 1998, Peñuelas and Estiarte 1998, Lindroth 2010). Elevated CO$_2$ usually results in increases in tannins and sometimes results in increases in low molecular weight phenolics (Koricheva et al. 1998, Stiling and Cornelissen 2007, Lindroth 2010). There is little or no change in concentrations of terpenes (Koricheva et al. 1998, Stiling and Cornelissen 2007). In part, the high variability of chemical responses of plants to elevated CO$_2$ are due to responses that are specific to taxon and/or to interactive effects specific to various environments (Lindroth 2010).
Ozone is a highly reactive compound that adversely affects plants. A recent meta-analysis indicates that current levels of O₃ have reduced tree biomass by 7% compared to pre-industrial levels (Wittig et al. 2009). Ozone enters plants through leaf stomata, diffuses into the leaves, and reacts with lipids and proteins contained in cell wall and plasma membranes (Fuhrer and Booker 2003, Kangasjarvi et al. 2005). The interaction of O₃ with these cellular components produces aldehydes, peroxides, and reactive oxygen species, which may cause cell death, induce stomatal closure, or alter metabolic processes (Fuhrer and Booker 2003, Kangasjarvi et al. 2005, Valkama et al. 2007).

Trees exposed to elevated ozone may reduce carbon fixation and photosynthesis, shift carbon/nutrient allocation patterns, and alter leaf and root transpiration (Chappelka and Samuelson 1998, Samuelson and Kelly 2001). Ozone has also been shown to slow stomatal responses to light (Paoletti 2005), water stress (Maier-Maercker 1999), and closure at night (Matyssek et al. 1995, Grulke et al. 2004), as well as decrease allocation to fine root production. If realized, these changes could increase plant susceptibility to drought, resulting in increased plant stress (Samuelson and Kelly 2001).

Chronic exposure to elevated O₃ can also modify plant chemical composition. Because ozone affects stomatal physiology and therefore photosynthesis, the flow of carbon to primary and secondary metabolic pathways can shift. Ozone also induces antioxidant defenses, which are linked to the shikimic acid pathway (Valkama et al. 2007). This upregulation may explain the observed increase in levels of some secondary metabolites, predominantly phenolics and terpenes, in plants exposed to elevated O₃ (Valkama et al. 2007, Lindroth 2010). However, induced changes in concentrations of secondary metabolites exposed to elevated O₃ can be modulated by pathogen and
mycorrhizal infection of plants (Bonello et al. 1993).

**Effects of elevated CO\(_2\) and O\(_3\) on plant-insect interactions**

Elevated CO\(_2\) does not directly affect insects (Coviella and Trumble 1999), but elevated O\(_3\) can directly affect insects by interacting with and degrading biogenic volatile organic carbons, which are olfactory cues utilized by herbivores and natural enemies in locating hosts (Laothawornkitkul et al. 2009, Yuan et al. 2009). Elevated O\(_3\) can also influence host selection for oviposition (Jones and Coleman 1988, Kopper and Lindroth 2003b). Nonetheless, both CO\(_2\) and O\(_3\) are largely expected to influence insects indirectly by changes in plant quality (Peñuelas and Estiarte 1998, Lindroth 2010).

Changes in plant quality due to exposure to elevated CO\(_2\) can modify insect herbivore preference, performance, and survival (Peñuelas and Estiarte 1998). Insects feeding on CO\(_2\)-enriched foliage typically increase food consumption, likely to compensate for reduced N content of leaves. However, the ability of insects to compensate may be limited in some cases by increased concentrations of secondary metabolites (Lindroth 2010). In general, insect performance is altered by protracted developmental rates and reduced adult size and fecundity (Peñuelas and Estiarte 1998, Awmack et al. 2004). Contrastingly, although plants growing under elevated O\(_3\) generally exhibit higher concentrations of phenolics and terpenes, herbivores feeding on O\(_3\)-enriched plants generally exhibit reduced development times and higher pupal masses, which indicates that host quality is improved (Valkama et al. 2007). However, these responses to CO\(_2\) and O\(_3\) are not universal and are limited by context, the species
involved, and environmental factors. Ultimately, changes in insect preference and
performance in a CO$_2$ or O$_3$ enriched environment could alter host selection and
population dynamics of herbivores (Peñuelas and Estiarte 1998, Lindroth and Dearing
2005).

A meta-analysis of 63 studies indicates that when levels of CO$_2$ and O$_3$ are
elevated simultaneously, many of the positive effects of O$_3$ on insect performance are
negated (Valkama et al. 2007). However, as Lindroth (2010) points out, this metanalysis
did not differentiate between additive and interactive effects. Studies to date primarily
indicate that CO$_2$ and O$_3$ interact additively as no interactions were detected between CO$_2$
and O$_3$ on performance of aphids (Awmack et al. 2004, Peltonen et al. 2006), forest tent
caterpillars (Agrell et al. 2005), leaf miners (Kopper and Lindroth 2003) or whitemarked
tussock moths (Kopper et al. 2001).

**Additional factors of climate change affecting plant-insect interactions**

Changes in CO$_2$ and O$_3$ are just two factors that are currently changing due to
anthropogenic activities. Increases in global temperature and altered precipitation will
also impact plant-insect interactions and will likely modify many of the previously
described effects of CO$_2$ and O$_3$. The relationship between plants and insects under future
climatic conditions are difficult to predict. Global temperature is anticipated to increase
by 1.8–4.0$^\circ$C during the 21$^{st}$ century (IPCC 2007). Current patterns of insect diversity
and abundance increase from cold to warm latitudes suggesting a broad relationship with
temperature (Wilf and Labandeira 1999). Additionally, because insects are poikilotherms,
their development and growth rates will accelerate as temperatures increase.

A good indicator of future interactions can be found by reconstructing interactions that occurred 55.8 million years ago during the Paleocene-Eocene thermal maximum, which was a time marked by a sudden increase in CO₂ and temperature. During a 10,000 year time span, temperatures increased by approximately 5°C and concentrations of CO₂ tripled (Zachos et al. 2003). At the same time fossil evidence indicates rapid shifts in geographic distributions of insects, increased herbivory (from 38 to 57 percent leaves damaged), and increased insect diversity and abundance (Currano et al. 2008).

Droughts have long been associated with tree mortality at regional scales throughout the vegetated continents. Anthropogenic-driven climate change is predicted to increase drought frequency, duration, and severity (IPCC 2007), which will likely increase the severity and frequency of insect outbreaks (McDowell et al. 2008, Allen et al. 2010). The mechanisms underlying drought-induced tree mortality have been poorly understood, but McDowell et al. (2008) proposed three mechanisms of drought-induced forest mortality, which are not mutually exclusive: (1) hydraulic failure, (2) carbon starvation, and (3) biotic agent demographics. Hydraulic failure can operate when extreme drought kills trees via cavitation of water columns in the xylem (Rennenberg et al. 2006, Zweifel and Zeugin 2008). Carbon stress can occur when chronic water stress causes plant carbon deficits (McDowell et al. 2008, Adams et al. 2009, Breshears et al. 2009), possibly resulting in altered resistance to insects and pathogens. Finally, elevated heat, which is frequently associated with drought events, can increase survival and accelerate increases in populations of insects and pathogens, allowing them to overwhelm stressed hosts (Desprez-Loustau et al. 2006, Raffa et al. 2008, Wermelinger et al. 2008).
Hypotheses and Objectives

The **goals** of my dissertation research were to characterize mechanisms of birch resistance to bronze birch borer, their relationship to whole plant physiology, and how their expression is modified by environmental stress. This was accomplished by comparing constitutive and induced resistance mechanisms of paper birch against bronze birch borer to those of susceptible European white birch (*Chapters 2 and 3*); by girdling paper birch to disrupt transport of photosynthate and alter within-tree resistance to bronze birch borer (*Chapter 4*); and by analyzing how elevated atmospheric CO\textsubscript{2} and O\textsubscript{3} affect paper birch resistance to bronze birch borer (*Chapter 5*).

My **central hypotheses** were that paper birch, due to its coevolutionary history with bronze birch borer, employs constitutive and/or induced chemical/physical defenses targeted to bronze birch borer that are not present in European white birch, which lacks this shared history. Secondly, birch resistance to bronze birch borer is dependent on transport of current photosynthate from leaves to the trunk. Finally, elevated levels of CO\textsubscript{2} will augment defenses of paper birch while elevated levels of O\textsubscript{3} will diminish defenses to bronze birch borer. To test these hypotheses, I used the following **experimental objectives**: (1) to characterize and compare constitutively expressed phenolics in phloem tissues of paper birch to European white birch (*Chapter 2*); (2) to compare induced responses (i.e. rate of wound periderm formation and phytochemistry) in paper birch to European white birch (*Chapter 3*); (3) to experimentally disrupt transport of current photosynthate by girdling paper birch and then compare wound
periderm growth, defensive chemistry, and bronze birch borer colonization above (resistant) and below (susceptible) the girdle in paper birch over time (Chapter 4); and (4) to quantify the effects of elevated and ambient levels of CO₂ and O₃ alone and in combination on wound periderm formation, phytochemistry, and bronze birch borer colonization in paper birch (Chapter 5).
References


preference or growth performance of insect herbivores in transgenic silver birch (Betula pendula Roth)? Planta 222: 699-708.


White, T. C. R. 1993. The inadequate environment: nitrogen and the abundance of animals. Springer-Verlag, New York, New York, USA.


Chapter 2

Inter- and intra-specific variation in constitutive phloem chemistry of paper birch 
(Betula papyrifera) and European white birch (Betula pendula)

Abstract

In woody angiosperms, there is both interspecific and intraspecific variation resistance to wood-boring insects. Bronze birch borer (Agrilus anxius), a beetle endemic to North America, has been associated with mortality of birches (Betula), although there is substantial variation in resistance among species and within species. Paper birch (B. papyrifera), which is indigenous to North America, shares an evolutionary history with bronze birch borer and is resistant to colonization. In contrast, European white birch (B. pendula) has not interacted with bronze birch borer and is much more susceptible. In a search for possible mechanisms of birch resistance to bronze birch borer, we compared the constitutive phenolic chemistry of phloem tissues from resistant B. papyrifera with susceptible B. pendula. Secondly, we analyzed the genotypic variation in phenolic composition among clones and/or half-sibs of each of these species. There were qualitative and quantitative differences in phenolic composition between species, as indicated by univariate and multivariate analyses. Three phenolics (coumaroylquinic acid, betuloside pentoside A, and a diarylheptanoid hexoside) were detected only in B.
*papyrifera.* Additionally, concentrations of total phenolics and six individual phenolics were significantly higher in *B. papyrifera.* These differences might contribute to resistance of *B. papyrifera* to bronze birch borer. There was significant intraspecific variation in 24% of analyzed phenolics in *B. papyrifera* clones and half-sibs, and 36% in *B. pendula* clones, but clones and half-sibs within each species could not be distinguished by phenolic composition using multivariate analysis.
Introduction

In woody angiosperms, there is variation in both interspecific (Morewood et al. 2004, Rebek et al. 2008) and intraspecific resistance (Miller et al. 1991) to wood-boring insects, and this variation can be modulated by environmental stress (Anderson 1944, Dunn et al. 1990). Bronze birch borer (*Agrilus anxius* Gory), a beetle indigenous to North America, has been associated with mortality of birches (*Betula* spp.), although there is considerable variation in resistance among species (Ball and Simmons 1980, Santamour 1982, Miller et al. 1991, Herms 2002) and within species (Miller et al. 1991). In forest systems, periodic outbreaks have been associated with stressed birch, resulting in widespread mortality (Balch and Prebble 1940, Jones et al. 1993). Damage by bronze birch borer occurs when larvae feed on phloem tissue, producing galleries (Barter 1957). Galleries disrupt transport of photosynthate, which can girdle and kill trees (Barter 1957).

Relatively little is known about the physical and chemical traits that underlie resistance of woody angiosperms against wood-boring insects. Proposed physical mechanisms of resistance include high sap flow (Morewood et al. 2004) and high bark moisture (Hanks et al. 1991, Hanks et al. 1999), both of which can inhibit larval establishment. Additionally, the formation of wound periderms (callus tissues) are hypothesized to function defensively by encapsulating feeding larvae (Anderson 1944, Dunn et al. 1990, Fierke and Stephen 2008). Putative chemical mechanisms include the presence of uncharacterized compounds in the bark or phloem tissues (Morewood et al. 2004), and phenolics (Eyles et al. 2007), which are secondary metabolites that can have a defensive function (Julkunen-Tiitto et al. 1996, Buer et al. 2010).
Historically, wood-borers have been typically categorized as secondary pests that preferentially colonize stressed trees (Balch and Prebble 1940, Anderson 1944, Dunn et al. 1986, Jones et al. 1993, Stephen et al. 2001). However, with the introduction of exotic wood-boring beetles to North America, wood-borers have recently been additionally categorized as killers of healthy trees (Poland and McCullough 2006, Hu et al. 2009). Although not a universal pattern, naïve host plant populations and species that lack coevolutionary histories with an herbivore can be highly susceptible to successful colonization and feeding injury by herbivores, which can lead to plant mortality (Witter and Ragenovich 1986, Houston 1994, Havill et al. 2006, Rebek et al. 2008, Gandhi and Herms 2010). However, plants that have an evolutionary history of interaction with an herbivore can be much more resistant (Anderson 1944, Hanks et al. 1999), possibly due to the presence of targeted defenses that have arisen during selection imposed by herbivores.

The interaction between bronze birch borer, which is a wood-boring beetle native to North America, and birch is consistent with this pattern (Miller et al. 1991, Nielsen et al. in press). Betula spp. indigenous to North America, such as paper birch (B. papyrifera Marshall), share an evolutionary history with bronze birch borer and are highly resistant to successful colonization by larvae unless stressed by drought, injury, temperature, or advanced age (Balch and Prebble 1940, Anderson 1944, Barter 1957, Jones et al. 1993). The physiological changes underlying the shift from a resistant to susceptible state are not understood. In contrast, tree species exotic to North America, such as European white birch (B. pendula Roth) that do not share an evolutionary history with birch borer, are highly susceptible to bronze birch borer (Ball and Simmons 1980, Miller et al. 1991).
The secondary chemistry of Betula spp. is dominated by compounds containing phenols, including low molecular weight phenolics, tannins and lignin (Julkunen-Tiitto et al. 1996, Ossipov et al. 1996, Mämmelä 2001, Laitinen et al. 2004, Mattson et al. 2005). Phenolics can have a wide range of functions, but a primary function is as a defense against insects and pathogens (Harborne 2001, Barbehenn et al. 2006, Buer et al. 2010). Therefore, the first goal of this study was to compare interspecific variation of constitutively expressed low-molecular weight phenolics present in phloem tissues of B. papyrifera and B. pendula and to assess how this variation corresponds with known resistance to bronze birch borer. The second goal of this study was to analyze intraspecific variation in constitutively expressed phenolics among four clones or half-sibs within each of these species.

Methods

Common garden study site and experimental design

Betula papyrifera and B. pendula used in this study have been growing in a common garden experiment for 12 years at The Ohio State University’s Agricultural Research and Development Center in Wooster, Ohio, USA. Trees were planted at a 3 m X 3 m spacing. In total, there are 56 B. papyrifera and 54 B. pendula. Betula papyrifera and B. pendula had mean stem heights of 35.3±1.3 (SE) and 46.5±1.2 m, and mean stem diameters of 9.1±0.3 and 10.4±0.3 cm, respectively.
Intraspecific variation of *B. papyrifera* was represented by two clones (231 and M2) and two half-sib families (M1 and 12). M1, M2, and 12 were collected from Midland County Michigan and 231 was collected from Pennsylvania. Intraspecific variation of *B. pendula* was represented by four clones (5818, 5832, 5938 and 50076). *Betula pendula* clones are used in birch cultivation by the Finnish forest industry and have been previously evaluated for their resistance to mammalian and insect herbivores (Rousi et al. 1997, Ylioja et al. 2002).

Trees were planted in a completely randomized block design with eight trees of each species per block, two replicates per clone. One tree of each of the four clones per species was randomly sampled in each of the eight blocks (N=64). Clone was treated as a nested factor within species.

*Phloem samples*

One phloem sample was excised from each tree stem using a 10 mm arch punch at approximately 1.3 m above ground level. Samples were taken 26 June 2007 to correspond with the natural phenology of neonate feeding by bronze birch borer (Akers and Nielsen 1984). Upon excision, samples were immediately flash frozen in liquid nitrogen. The tissue was ground to a powder in liquid nitrogen and 100 mg (fresh weight) was used for extraction of phenolics. The ground tissue was extracted twice in 500 µL of HPLC grade methanol (Fisher, Pittsburgh, PA) for 24 h at 4°C (Bonello and Blodgett 2003). The supernatants were combined and stored in a 1.5-ml microcentrifuge tube at -20°C until analysis by high-performance liquid chromatography (HPLC).
Qualitative phenolic analysis

To characterize constitutively expressed phenolics, HPLC-MS analysis was completed using a Varian 212-LC chromatography pump, Varian Polaris™ RP18, 4.6 X 150 mm column, ProStar 410 HPLC Autosampler, and Varian 500-MS ion trap mass spectrometer with the ESI source operated in the negative ion mode. The MS detector had a capillary voltage of 80 V and a cone voltage of 5000 V, with a scan range of m/z 100-1400 m/z. MS² scans were completed on-the-fly by the instrument when the threshold of the pseudomolecular parent ion reached 20,000 counts. Ions generated by MS² and MS³ underwent further fragmentation at 4000 counts. The binary mobile phase consisted of 0.1% glacial acetic acid in HPLC grade water (solvent A) and 0.1% glacial acetic acid in HPLC grade methanol (solvent B). The flow rate was 0.300 mL/min with a linear gradient of B from 0 to 10% in10 min, then to 25% by 20 min, to 35% by 40 min, to 45% by 56 min, to 65% by 64 min, and finally to 100% by 70 min, with a 20 min hold. The injection volume for each sample was 5 µL.

Mass spectra for each compound were confirmed using a Waters 2695 separations module (Milford, MA) equipped with a Phenomenex Kinetex™ RP18, 2.6 µm, 3.00 X 150 mm column, a Phenomenex 0.5 µm X 0.0102 mm in Ultra Column In-Line Filter, a Waters 996 photodiode array detector and a Micromass ZQ 2000 quadrupole detector with an ESI source operated in negative ion mode. The photodiode array detector scanned between 237 nm and 400 nm wavelengths, with peak areas calculated using absorbance at 280 nm. The MS detector had a capillary voltage of 3.2 kV, a cone voltage of 60 V, and...
a source temperature of 80° C, with a scan range of m/z 100-1200. The autosampler and column temperatures were set at 30° C. Samples were run twice. The first run utilized the previously described binary mobile phase and solvent program. The second run utilized a 1:2 splitter and the binary mobile phase and solvent program used for phenolic quantification as described below.

Quantitative phenolic analysis

Quantification of phenolics was carried out using a Waters 2690 separations module (Milford, MA) equipped with a Waters Xterra™ RP18, 5 µm, 4.6 X 150 mm column, a Waters Xterra™ RP18, 3.9 µm, 3.0 X 20 mm guard column, and a Waters 996 photodiode array detector. The binary mobile phase consisted of 2% glacial acetic acid in HPLC grade water (solvent A) and 2% glacial acetic acid in HPLC grade methanol (solvent B). The flow rate was 1 mL/min, with a linear gradient of B: 0% (0-2 min), 0-10% (2-4 min), 10-48% (4-20 min), 48-100% (20-38 min), and then held for 1 min. The injection volume for each sample was 10 uL.

Phenolics were quantified against the following standards: catechin (Alexis Biochemicals, San Diego, CA, USA), chlorogenic acid (MP Biomedicals, Solon, OH, USA), and salicin (Sigma Aldrich, Milwaukee, WI, USA). The glycosides catechin pentoside, betuloside, betuloside pentosides, and diarylheptanoids were expressed as salicin equivalents. Catechin and procyanidins were expressed as catechin equivalents. Coumaroylquinic acid, platyphyllloside and unknown peaks 9-12 were expressed as chlorogenic acid equivalents.
Statistical analyses

For statistical analysis, all factors were considered fixed. Rate of wound closure and the relative abundance of phenolic compounds were analyzed by univariate analysis of variance (ANOVA) using SAS 9.1 (SAS Institute, 1999). Data that did not have equal variances were log-transformed to meet this assumption. Fisher’s LSD was used to separate means of clones (α = 0.05). Multivariate analyses were conducted using principal component analysis (PCA) using Minitab 15 (Minitab Inc, 2007) to compare intraspecific and interspecific variation of phenolic profiles.

Results

Characterization of phenolics

Compounds that were present in high quantities or were presently consistently in at least 50% of the analyzed samples per species were selected for chemical characterization. Based on these criteria, 17 phenolics were characterized (Fig. 2.1). Tentative identification of chemicals was based on comparison of UV absorption, mass fragmentation, and relative retention time, with published data and standards, when available (Table 2.1). Fragmentation patterns generated from the quadrupole mass spectrometer are described when different from those generated by the ion trap mass spectrometer.
Figure 2.1. Comparison of constitutive phenolic profiles from phloem of A paper birch (*Betula papyrifera*) and B European white birch (*Betula pendula*). Traces were generated from the HPLC-PDA at 280 nm. For characterization of numbered peaks, see Table 2.1.
Table 2.1. Characterization of constitutive phenolic chemistry of phloem of paper birch (*Betula papyrifera*) and European white birch (*Betula pendula*) using ion trap mass spectrometry.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Rt (min)</th>
<th>[M-H] (m/z)</th>
<th>MS&lt;sup&gt;2&lt;/sup&gt; Ions</th>
<th>MS&lt;sup&gt;3&lt;/sup&gt; Ions</th>
<th>MS&lt;sup&gt;4&lt;/sup&gt; Ions</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Tentative Identification</th>
<th>Presence in Tree Species</th>
<th>References used for Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.4</td>
<td>577.3</td>
<td>425.2, 451.1, 289.3</td>
<td>407.1</td>
<td>281.1, 283.1, 255.1</td>
<td>280</td>
<td>procyanidin dimer</td>
<td>both</td>
<td>Tsang et al. 2005</td>
</tr>
<tr>
<td>2</td>
<td>20.0</td>
<td>421.2</td>
<td>289.1, 245.1, 205.1</td>
<td>203.1</td>
<td></td>
<td>279</td>
<td>catechin pentoside</td>
<td>both</td>
<td>Männmelä 2001</td>
</tr>
<tr>
<td>3</td>
<td>20.7</td>
<td>289.1</td>
<td>245.1, 203.0, 187.1</td>
<td>175.0, 187.9, 161.0</td>
<td></td>
<td>280</td>
<td>catechin</td>
<td>both</td>
<td>Männmelä 2001</td>
</tr>
<tr>
<td>4</td>
<td>21.6</td>
<td>865.4</td>
<td>695.4, 577.4, 738.9</td>
<td>543.2, 451.3</td>
<td></td>
<td>278, 283sh</td>
<td>procyanidin trimer</td>
<td>both</td>
<td>Tsang et al. 2005</td>
</tr>
<tr>
<td>5</td>
<td>25.4</td>
<td>337.1</td>
<td>163, 118.9, 92.9</td>
<td></td>
<td></td>
<td>284sh, 311</td>
<td>coumaroylquinic acid</td>
<td>B. papyrifera</td>
<td>Poon et al. 1998</td>
</tr>
<tr>
<td>6</td>
<td>28.2</td>
<td>459.3</td>
<td></td>
<td></td>
<td></td>
<td>278</td>
<td>betuloside pentoside A</td>
<td>B. papyrifera</td>
<td>Männmelä 2001</td>
</tr>
<tr>
<td>7</td>
<td>28.7</td>
<td>327.2</td>
<td></td>
<td></td>
<td></td>
<td>278</td>
<td>betuloside</td>
<td>both</td>
<td>Smite et al. 1993; Männmelä 2001</td>
</tr>
<tr>
<td>8</td>
<td>29.9</td>
<td>459.3</td>
<td>327.2, 165.1</td>
<td>121.0, 106.0</td>
<td></td>
<td>278</td>
<td>betuloside pentoside B</td>
<td>both</td>
<td>Männmelä 2001</td>
</tr>
<tr>
<td>9</td>
<td>36.7</td>
<td>487.2</td>
<td></td>
<td></td>
<td></td>
<td>280</td>
<td>unknown</td>
<td>both</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>39.2</td>
<td>427.2</td>
<td>293.1, 125.0, 233.1, 191.0</td>
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<td>280</td>
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<td>11</td>
<td>40.5</td>
<td>311.2</td>
<td>205.1, 120.9, 146.9</td>
<td>92.9</td>
<td></td>
<td>280</td>
<td>unknown</td>
<td>both</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>45.6</td>
<td>607.4</td>
<td>310.6, 477.3</td>
<td></td>
<td></td>
<td>280</td>
<td>unknown</td>
<td>both</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>46.7</td>
<td>475</td>
<td></td>
<td></td>
<td></td>
<td>278, 283sh</td>
<td>platyphylloside</td>
<td>both</td>
<td>Smite et al. 1993; Männmelä 2001</td>
</tr>
<tr>
<td>14</td>
<td>48.2</td>
<td>477.3</td>
<td>315.3, 149.1, 147.1, 297.1</td>
<td>107.0, 121.0, 92.9</td>
<td>278, 283sh</td>
<td>diarylheptanoid hexoside</td>
<td>B. papyrifera</td>
<td>Jiang 2006</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>63.5</td>
<td>725.5</td>
<td>593.5, 461.6, 461.4</td>
<td>299.3</td>
<td></td>
<td>278, 283sh</td>
<td>centrolobol + hexose + pentose + pentose</td>
<td>both</td>
<td>Smite et al. 1993</td>
</tr>
<tr>
<td>16</td>
<td>64.1</td>
<td>593.4</td>
<td>461.4, 299.5, 299.3</td>
<td>177.2, 121.1</td>
<td></td>
<td>278, 283sh</td>
<td>centrolobol + hexose + pentose</td>
<td>both</td>
<td>Smite et al. 1993</td>
</tr>
<tr>
<td>17</td>
<td>64.7</td>
<td>461.4</td>
<td>299.3, 177.1, 121.1</td>
<td>121.0, 106.0, 159.1</td>
<td></td>
<td>278, 283sh</td>
<td>centrolobol hexoside</td>
<td>both</td>
<td>Smite et al. 1993</td>
</tr>
</tbody>
</table>

*Bold indicates ions that underwent further fragmentation.*
Peak 1 had a [M-H]– at m/z 577.3 with MS² yielding major fragment ions at m/z 425.2, 451.1, and 289.3. The m/z 425.2 fragment ion underwent MS³ yielding a major fragment at m/z 407.1 and MS⁴ at 281.1, 283.1, 255.1. The first fragment ion at m/z 425.2, a loss of 152 amu, is consistent with the retro-Diels-Alder fission (RDA-F) and the m/z 407.1 is consistent with RDA-F after interflavanic bond cleavage (Friedrich et al. 2000, Shui and Leong 2004). These spectral characteristics are consistent with those reported for a procyanidin dimer (Tsang et al. 2005).

Peak 2 had a [M-H]– at m/z 421.2 with MS² yielding a major fragment ion at m/z 289.1, and MS³ at m/z 245.1 and 205.1. The m/z 245.1 ion underwent MS⁴ yielding m/z 203.1. The major fragment m/z 289.1, a loss of 132 amu, indicates the loss of one pentose. These fragment ions correspond with those reported for catechin pentoside found in European white birch wood (Mämmelä 2001).

Peak 3 had a [M-H]– at m/z 289.1 with MS² yielding a major fragment ion at m/z 245.1, and MS³ at m/z 203.0, and 187.1. The m/z 203.0 fragment ion underwent MS⁴ yielding m/z 175.0, 187.9, and 161.0. This compound was identified as catechin by comparison with a standard.

Peak 4 had a [M-H]– at m/z 865.4 with MS² yielding major fragment ions at m/z 695.4 and 577.4. The m/z 695.5 fragment ion underwent MS³ yielding m/z 543.2 and 451.3. The first fragment ion at m/z 695.4, a loss of 170 amu, is consistent with RDA-F with an additional loss of water. The second ion at m/z 577.4, which represents a loss of 288 amu, is consistent with interflavanic bond cleavage (Friedrich et al. 2000, Shui and Leong 2004). These spectral characteristics are consistent with those reported for a procyanidin trimer (Tsang et al. 2005).
Peak 5 had a [M-H]− at m/z 337.1 with MS² yielding a major fragment ion at m/z 163.0, MS³ at m/z 118.9, and MS⁴ at 92.9. The pseudomolecular ion m/z 337.1 is consistent with coumaroylquinic acid (Poon 1998). The UV maximum is consistent with that found in European white birch leaves (Ossipov et al. 1996).

Peaks 6 and 8 had a [M-H]− at m/z 459.3. Peak 6 underwent further fragmentation with MS² yielding a major fragment ion at m/z 327.2, MS³ at m/z 165.1, and MS⁴ at m/z 121.0 and 106.0. The first fragment ion at m/z 327.2, a loss of 132 amu, indicates a loss of a pentose. The remaining aglycone (m/z 327.2) is consistent with betuloside, suggesting this compound is betuloside pentoside (Mämmelä 2001). Peaks 6 and 8 had the same fragmentation pattern in the quadrupole mass spectrometer suggesting that they are enantiomers. Therefore, they were tentatively identified as betuloside pentoside A and betuloside pentoside B.

Peak 7 had a [M-H]− at m/z 327.2. This MW is consistent with the pseudomolecular ion for betuloside (Mämmelä 2001).

Peak 9 had a [M-H]− at m/z 487.2. There was no further fragmentation.

Peak 10 had a [M-H]− at m/z 427.2 with MS² yielding a major fragment ion at m/z 293.1, MS³ at m/z 125.0, 233.1 and 191.0. Fragment ion m/z 125.0 underwent MS⁴ resulting in m/z 97.0.

Peak 11 had a [M-H]− at m/z 311.2 with MS² yielding a major fragment ion at m/z 205.1, MS³ at m/z 120.9 and 146.9. Fragment ion m/z 120.9 underwent MS⁴ resulting in m/z 92.9.

Peak 12 had a [M-H]− at m/z 607.4. with MS² yielding a major fragment ion at m/z 310.6 and 477.3
Peak 13 had a [M-H]− at m/z 475.0. There was no further fragmentation generated by the ion-trap mass spectrometer. However, there was fragmentation of the parent ion in the quadrupole mass spectrometer generating a base ion at m/z 295. This loss of 180 amu is indicative of hexose + water, which in combination with the resulting aglycone m/z 295 are consistent with platyphyllloside (Vainiotalo et al. 1991, Smite et al. 1993) (Fig. 2.2).

Peak 14 had a [M-H]− at m/z 477.3 with MS² yielding a major fragment ion at m/z 315.3, MS³ at m/z 149.1, 147.1, and 297.1. The m/z 149.1 fragment ion underwent MS⁴ yielding m/z 107.0, 121.0, and 92.9. The first fragment ion m/z 315.3, a loss of 162 amu, indicates a loss of a hexose. Major fragments m/z 149.1 and 121.0 and the aglycone m/z 315.3 are consistent with a diarylheptanoid related to platyphyllloside (Jiang et al. 2006). Therefore, this compound was tentatively identified as diarylheptanoid hexoside (Fig. 2.2).

Peaks 15, 16, and 17 shared the same aglycone (m/z 299.3), but had different sugar moieties as described below (Fig. 2.2). The m/z 121 fragment generated from fragmentation of the aglycone is consistent with the presence of an ethyl p-hydroxybenzoyl moiety, which is indicative of a diarylheptanoid (Jiang et al. 2006). The aglycone (m/z 299.3) is consistent with with the diarylheptanoid centrolobol (Smite et al. 1993). This compound has previously been detected in B. pendula phloem and conjugated with a monosaccharide, disaccharide, and trisaccharide comprised of glucose and apiose (Smite et al. 1993).

Peak 15 had a [M-H]− at m/z 725.5 with MS² yielding major fragment ions at m/z 593.5 and 461.6. The m/z 593.5 fragment ion underwent MS³ yielding m/z 461.4 and MS⁴ yielding m/z 299.3. The first fragment ion m/z 593.5, a loss of 132 amu, indicates a loss
Figure 2.2. Proposed identity of HPLC peaks (Fig. 1). **A** Platiphyllloside (peak 13). **B** diarylheptanoid hexoside (peak 14). **C** centrolobol glycosides containing three, two and one sugar moieties (peaks 15, 16, 17, respectively).
of a pentose. The second fragment ion $m/z$ 461.4, a loss of 132 amu from the MS$^2$ ion, indicates a second loss of pentose. The third fragment ion $m/z$ 299.3, a loss of 162 amu from the MS$^3$ ion, indicates a loss of a hexose. In summary, fragmentation patterns are consistent with centrolobol conjugated with a hexose and two pentose sugars.

Peak 16 had a [M-H]$^-$ at $m/z$ 593.4 with MS$^2$ yielding major fragment ions at $m/z$ 461.4 and 299.5. The $m/z$ 461.4 fragment ion underwent MS$^3$ yielding $m/z$ 299.3 and MS$^4$ yielding $m/z$ 177.2 and 121.1. The first fragment ion $m/z$ 461.4, a loss of 132 amu, indicates a loss of a pentose. The second fragment ion $m/z$ 299.3, a loss of 162 amu from the MS$^2$ ion, indicates a loss of a hexose. In summary, fragmentation patterns are consistent with centrolobol conjugated with a hexose and a pentose sugar.

Peak 17 had a [M-H]$^-$ at $m/z$ 461.4 with MS$^2$ yielding a major fragment ion at $m/z$ 299.3 and MS$^3$ at $m/z$ 177.1 and 121.1. The $m/z$ 177.1 fragment ion underwent MS$^4$ yielding $m/z$ 121.0, 106.0, and 159.1. The first fragment ion $m/z$ 299.3, a loss of 162 amu, indicates a loss of a hexose. Therefore, fragmentation patterns are consistent with centrolobol hexoside.

*Interspecific and intraspecific variation in phenolics*

Coumaroylquinic acid (5), betulose pentoside A (6), and diarylheptanoid hexoside (14) were detected only in *B. papyrifera*. Betulose pentoside B (8) ($F=20.86$), centrolobol glycoside (15) ($F=20.72$), and four unknown compounds (9, 10, 11, 12) ($F=38.87, 68.97, 32.44$, and $8.74$, respectively) were significantly higher in *B. papyrifera*, while catechin pentoside (2) ($F=62.97$), platyphyloside (13) ($F=12.26$), and
centrolobol glycoside (16) \((F=31.99)\) were higher in \emph{B. pendula}. Concentrations of total phenolics (as detected by HPLC) were higher in \emph{B. papyrifera} \((F=4.53)\) (Table 2.2).

There were no intraspecific differences in 13 of the 17 phenolics in \emph{B. papyrifera} and in 9 of the 14 phenolics in \emph{B. pendula}. Amounts of catechin pentoside (2) \((F=5.26)\), procyanidin trimer (4) \((F=16.60)\), coumaroylquinic acid (5) \((F=4.49)\), and betuloside (7) \((F=9.14)\) were significantly different among clones of \emph{B. papyrifera} using ANOVA (Table 2.3). Amounts of catechin (3) \((F=3.42)\), procyanidin trimer (4) \((F=4.33)\), compound 12 \((F=3.29)\), and two centrolobol glycosides (15, 16) \((F=13.81, 5.18, \text{respectively})\) were significantly different among clones of \emph{B. pendula}.

There was distinct separation in multivariate phenolic chemistry between species along the first PC axis, which explained 29.6\% of the total variation (Fig. 2.3). The first two principal components explained 57.8\% of the total variation. Location along the first PC axis was primarily determined by coumaroylquinic acid (5) (eigenvector (EV): 0.388), diarylheptanoid hexoside (14) (EV: 0.391), and compound 10 (EV: 0.376), which were associated with \emph{B. papyrifera}, and catechin pentoside (2) (EV: -0.291) and centrolobol glycoside (16) (EV: -0.214), which were associated with \emph{B. pendula}. Within each species, clones could not be distinguished by principal component analysis of their phenolic profiles. However, the variation (in ordination space) in phenolic chemistry among trees of \emph{B. papyrifera} was greater than in \emph{B. pendula} (Fig. 2.3).
**Table 2.2.** Comparison of individual and total concentrations of phenolics (mg/g fresh weight ± SE) in stem phloem tissue of paper birch (*Betula papyrifera*) and European white birch (*Betula pendula*) as detected by HPLC-PDA.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound</th>
<th><em>B. papyrifera</em></th>
<th><em>B. pendula</em></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>procyanidin dimer</td>
<td>6.78 ± 0.30</td>
<td>6.52 ± 0.19</td>
<td>0.380</td>
</tr>
<tr>
<td>2</td>
<td>catechin pentoside</td>
<td>62.07 ± 4.97</td>
<td>108.9 ± 4.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>catechin</td>
<td>12.53 ± 0.75</td>
<td>13.44 ± 0.52</td>
<td>0.354</td>
</tr>
<tr>
<td>4</td>
<td>procyanidin trimer</td>
<td>10.88 ± 0.80</td>
<td>11.18 ± 0.85</td>
<td>0.941</td>
</tr>
<tr>
<td>5</td>
<td>coumaroylquinic acid</td>
<td>3.36 ± 0.30</td>
<td>ND(^a)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>betuloside pentoside A</td>
<td>12.23 ± 1.19</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>betuloside</td>
<td>28.74 ± 3.42</td>
<td>28.13 ± 1.25</td>
<td>0.617</td>
</tr>
<tr>
<td>8</td>
<td>betuloside pentoside B</td>
<td>35.18 ± 3.17</td>
<td>21.39 ± 1.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>9</td>
<td>unknown</td>
<td>4.71 ± 0.57</td>
<td>1.75 ± 0.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>unknown</td>
<td>2.62 ± 0.31</td>
<td>0.16 ± 0.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>11</td>
<td>unknown</td>
<td>2.22 ± 0.22</td>
<td>0.67 ± 0.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>12</td>
<td>unknown</td>
<td>2.27 ± 0.49</td>
<td>0.65 ± 0.3</td>
<td>0.005</td>
</tr>
<tr>
<td>13</td>
<td>platyphylloside</td>
<td>24.68 ± 1.16</td>
<td>29.70 ± 0.91</td>
<td>0.001</td>
</tr>
<tr>
<td>14</td>
<td>diarylheptanoid hexoside</td>
<td>71.09 ± 6.22</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>centrolobol glycoside</td>
<td>8.15 ± 0.94</td>
<td>3.92 ± 0.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>16</td>
<td>centrolobol glycoside</td>
<td>16.44 ± 0.82</td>
<td>24.15 ± 1.29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>17</td>
<td>centrolobol hexoside</td>
<td>14.86 ± 0.69</td>
<td>14.08 ± 1.10</td>
<td>0.523</td>
</tr>
<tr>
<td></td>
<td>total phenolics</td>
<td>318.8 ± 16.5</td>
<td>282.5 ± 10.4</td>
<td>0.039</td>
</tr>
</tbody>
</table>

\(^a\)ND: Not detected.
Table 2.3. Intraspecific variation in concentrations of constitutive phenolics (mg/g fresh weight ± SE) in phloem of paper birch (Betula papyrifera) and European white birch (Betula pendula) as detected by HPLC-PDA.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>B. papyrifera</th>
<th>B. pendula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>M2</td>
</tr>
<tr>
<td>1</td>
<td>procyanidin dimer</td>
<td>6.5 ± 0.4 a</td>
<td>6.0 ± 0.4 a</td>
</tr>
<tr>
<td>2</td>
<td>catechin pentoside</td>
<td><strong>43.8 ± 6.6 b</strong></td>
<td><strong>88.1 ± 9.7 a</strong></td>
</tr>
<tr>
<td>3</td>
<td>catechin</td>
<td>11.1 ± 1.0 a</td>
<td>11.1 ± 0.9 a</td>
</tr>
<tr>
<td>4</td>
<td>procyanidin trimer</td>
<td><strong>8.1 ± 0.8 b</strong></td>
<td><strong>9.4 ± 0.6 b</strong></td>
</tr>
<tr>
<td>5</td>
<td>coumaroylquinic acid</td>
<td>4.7 ± 0.6 a</td>
<td><strong>1.9 ± 0.5 b</strong></td>
</tr>
<tr>
<td>6</td>
<td>betuloside pentoside A</td>
<td>10.5 ± 2.0 a</td>
<td>11.9 ± 0.9 a</td>
</tr>
<tr>
<td>7</td>
<td>betuloside</td>
<td><strong>18.2 ± 1.7 b</strong></td>
<td><strong>23.6 ± 2.1 b</strong></td>
</tr>
<tr>
<td>8</td>
<td>betuloside pentoside B</td>
<td>30.8 ± 4.4 a</td>
<td>28.6 ± 4.0 a</td>
</tr>
<tr>
<td>9</td>
<td>unknown</td>
<td>4.0 ± 0.7 a</td>
<td>2.9 ± 0.5 a</td>
</tr>
<tr>
<td>10</td>
<td>unknown</td>
<td>2.5 ± 0.5 a</td>
<td>2.3 ± 0.4 a</td>
</tr>
<tr>
<td>11</td>
<td>unknown</td>
<td>2.0 ± 0.5 a</td>
<td>2.4 ± 0.4 a</td>
</tr>
<tr>
<td>12</td>
<td>unknown</td>
<td>1.6 ± 0.4 a</td>
<td>3.1 ± 1.7 a</td>
</tr>
<tr>
<td>13</td>
<td>platyphylloside</td>
<td>22.4 ± 2.2 a</td>
<td>29.0 ± 3.2 a</td>
</tr>
<tr>
<td>14</td>
<td>dihydrietanoid hexoside</td>
<td>77.2 ± 9.8 a</td>
<td>65.5 ± 9.1 a</td>
</tr>
<tr>
<td>15</td>
<td>centrolobol glycoside</td>
<td>6.9 ± 1.9 a</td>
<td>11.5 ± 0.9 a</td>
</tr>
<tr>
<td>16</td>
<td>centrolobol glycoside</td>
<td>16.7 ± 1.9 a</td>
<td>18.4 ± 1.6 a</td>
</tr>
<tr>
<td>17</td>
<td>centrolobol hexoside</td>
<td>15.3 ± 1.0 a</td>
<td>15.3 ± 1.9 a</td>
</tr>
<tr>
<td></td>
<td>total phenolics</td>
<td><strong>282 ± 20.8 b</strong></td>
<td><strong>331 ± 25 ab</strong></td>
</tr>
</tbody>
</table>

Means within a row followed by different letters are significantly different within each species by LSD (P<0.05).** Bold indicates significant differences among clones within a species.  
** ND: Not detected.**
Figure 2.3. Inter- and intraspecific variation in phenolic chemistry of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*) as indicated by principal component analysis (PCA). 5818, 5832, 5938 and 50076 are clones of European white birch. M2 and 231 are clones and M1 and 12 are half-sibs of paper birch. Percentage of total variability explained by first two axes is shown.
Discussion

*Betula papyrifera* and *B. pendula* had similar phenolic profiles (Fig. 2.1). Fourteen of the 17 phenolics analyzed in this study were present in both species (Table 2.1), which may reflect their close phylogenetic relationship within the subgenus *Betula* (Schenk et al. 2008). Nonetheless, there were distinct differences in phenolic profiles between species, as indicated by principal component analysis. Phloem tissues of *B. papyrifera* contained coumaroylquinic acid, betuloside pentoside A, and a diarylheptanoid related to platyphyllloside, which were not detected in *B. pendula*. Total phenolics and six additional compounds were found in higher concentrations (betuloside pentoside B, a centrolobol glycoside, and 4 unknown compounds) in *B. papyrifera*. Three compounds, catechin pentoside, platyphyllloside and a centrolobol glycoside, were found in higher concentrations in *B. pendula*.

In our study, clones and half-sibs within each species could not be distinguished by their phenolic composition using multivariate analysis. However, intraspecific genotypic variation occurred in 24% of individual phenolics among clones and half-sibs of *B. papyrifera* and 36% in *B. pendula*. If the phenolics we identified and quantified are major determinants of host suitability for bronze birch borer, these findings might suggest little variation among clones and half-sibs in resistance to bronze birch borer. However, research on different half-sibs of paper birch documented substantial intraspecific variation in resistance to bronze birch borer (Miller et al. 1991). Generally, genetics has been shown to be a major source of intraspecific variation in birch secondary metabolites, (Keinanen et al. 1999, Laitinen et al. 2000, Laitinen et al. 2004), although ontogeny and
environment can also affect secondary chemistry (Julkunen-Tiitto et al. 1996, Laitinen et al. 2005). For example, in *B. pendula*, high variability in foliar phenolics of mature birches and in bark phenolics and triterpenes of birch seedlings is driven by genotype rather than herbivory or environmental conditions (Keinanen et al. 1999, Laitinen et al. 2000, Laitinen et al. 2004). Intraspecific genetic variability in the proportions of secondary metabolites is hypothesized to slow herbivore adaptation to a single chemical profile (Keinanen et al. 1999).

All but one of the identified compounds detected in these *Betula* species contained catechin (1-4) or the related arylbutanoids (6-8) or diarylheptanoids (13-17). Catechin and its isomers are important monomeric units used to synthesize procyanidins (condensed tannins) (Hagerman and Butler 1991), which we also detected in birch phloem tissues. Diarylheptanoids, which are biologically active, possessing anti-inflammatory, anti-hepatotoxic and/or free radical scavenging activity, have been isolated from many plant genera including *Acer, Alnus, Betula, Curcuma, Juglans* and *Zingiber* (Claeson et al. 1994, Akazawa et al. 2006). Platiphyllloside and centrolobol, both of which are glycosidic diarylheptanoids, are common components of birch phloem (Smite et al. 1993, Julkunen-Tiitto et al. 1996). These compounds have been found to have an inhibitory effect on ruminant digestion in vitro and are considered to be part of the chemical defense against mammalian herbivores (Sunnerheim et al. 1988, Sunnerheim and Bratt 2004). Coumaroylquinic acids (5) are common metabolites in plants and are involved in the biosynthesis of phenylpropanoids (Steck 1968). Many of the identified phenolics detected in *B. papyrifera* or *B. pendula* phloem tissues have been previously detected in phloem tissues of birch stems (Julkunen-Tiitto et al. 1996, Mämmelä 2001,
Laitinen et al. 2004), but to our knowledge coumaroylquinic acid, the diarylheptanoid hexoside, and the procyanidin dimer and trimer were detected for the first time in this study.

Previous research has suggested that birch species that lack rhododendrol (an arylbutanoid) are resistant to bronze birch borer (Santamour 1990, Santamour and Lundgren 1997). For example, *B. nigra* L. (river birch) lacks this compound and is not considered a host of bronze birch borer (Santamour 1990). Rhododendrol naturally occurs in some birch species and is also generated by the hydrolysis of the glycosides containing this aglycone, like betuloside (also known as rhododendrin) and betuloside pentoside (Smite et al. 1993, Julkunen-Tiitto et al. 1996, Santamour and Lundgren 1997). Rhododendrol has been implicated as a short-range stimulus for oviposition by mated bronze birch borer females (Santamour 1990). We found that the highly resistant *B. papyrifera* contained more betuloside and betuloside pentoside than susceptible *B. pendula*. Therefore, although the presence of rhododendrol or glycosides containing rhododendrol may mediate oviposition behavior, the absence of rhododendrol or glycosides containing rhododendrol is not a consistent indicator for bronze birch borer resistance.

The low-molecular weight phenolics measured in this experiment are only one class of compounds that are present in birch phloem that could affect larval performance. High-molecular weight phenolics (tannins and lignins) (Wainhouse et al. 1990, Moilanen and Salminen 2008), defensive proteins (Poyla 2003, Dafoe et al. 2009), and nutritive components (amino acids, proteins, sugars, sterols, etc.) (Haack and Slansky 1987, Slansky 1992) are important mediators of other plant-insect interactions and could also
impact growth and survival of wood-boring insects. Furthermore, the goal of this study was to characterize constitutively expressed phenolics, but induced responses of birch to bronze birch borer may also be important components of resistance.

In summary, we found qualitative and quantitative variation in phenolic composition of phloem of *B. papyrifera*, which is highly resistant to bronze birch borer, and *B. pendula*, which is much more susceptible. Specifically, we found three compounds that were only present in *B. papyrifera* and that *B. papyrifera* had higher concentrations of total phenolics and six individual phenolics in comparison to *B. pendula*. However, *in vitro* or *in vivo* bioassays are necessary to measure if these differences confer resistance to *B. papyrifera* to bronze birch borer. We also found genetic-based intraspecific variation in phenolic composition within both species, but clones and half-sibs within each species could not be distinguished by their phenolic composition using multivariate analysis.

**Acknowledgements**

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References


Minitab Inc. 2007. Minitab 15 statistical software, State College, PA.


Chapter 3

Comparison of induced responses in phloem tissues of paper birch (Betula papyrifera) and European white birch (Betula pendula) to bronze birch borer

Abstract

Among birch species (Betula), there is substantial variation in resistance to bronze birch borer (Agrilus anxius), a wood-boring beetle indigenous to North America that is prone to periodic outbreaks. For example, paper birch (B. papyrifera), by nature of its evolutionary history with bronze birch borer, is resistant although increased susceptibility has been associated with environmental stress. In contrast, European white birch (B. pendula), which lacks a shared history, is much more susceptible. The mechanisms by which birch resist bronze birch borer are not understood. Previous research identified possible constitutive mechanisms of birch resistance, so the goal of this experiment was to compare the induced responses in phloem of paper birch to European white birch. Specifically, we compared concentrations of phenolics, tannins, lignin, free amino acids, carbon, and nitrogen as well as growth rate of wound periderm (callus) induced by mechanical wounding and by inoculation of bronze birch borer. We found that induced chemical profiles of paper birch and European white birch were distinct, largely due to differences in phenolic composition. Additionally, European white birch had higher
concentrations of total phenolics and tannins, indicating that total pools do not confer resistance to bronze birch borer. In both species, mechanical wounding and bronze birch borer homogenate decreased concentrations of amino acids, phenolics, and tannins but increased concentrations of nitrogen and lignin. Our results suggest that the induced phenolic profile of paper birch, which is different than the induced profile of European white birch, might contribute to resistance to bronze birch borer.
Introduction

Outbreaks by endemic and exotic wood-borers have caused widespread tree mortality in forested and urban landscapes of North America (Balch and Prebble 1940, Nowak et al. 2001, Stephen et al. 2001, Poland and McCullough 2006), which can have strong impacts on forest ecosystem structure and function. Bronze birch borer (*Agrilus anxius* Gory) (Coleoptera: Buprestidae), a wood-boring beetle endemic to North America, is associated with widespread mortality of birch (*Betula* spp.). As bronze birch borer larvae feed on phloem tissue they produce galleries (Barter 1957). Galleries disrupt transport of photosynthate, which can girdle and kill trees (Barter 1957). There is considerable interspecific variation in resistance of birch as hosts for bronze birch borer (Ball and Simmons 1980, Santamour 1982, Miller et al. 1991, Herms 2002, Nielsen et al. *in press*). Paper birch (*B. papyrifera* Marshall) and other North American species share an evolutionary history with the beetle and are highly resistant unless stressed (Balch and Prebble 1940, Anderson 1944, Ball and Simmons 1980, Nielsen et al. *in press*). In contrast, European and Asian species, which lack a shared history with bronze birch borer, are much more susceptible (Ball and Simmons 1980, Miller et al. 1991, Herms 2002, Nielsen et al. *in press*).

Physical and chemical mechanisms, of which many can be constitutively expressed and/or induced by larval feeding, have been proposed to underlie resistance of woody angiosperms to wood-boring insects. High constitutive production of sap flow (Morewood et al. 2004) and high bark moisture (Hanks et al. 1991, Hanks et al. 1999) may physically impede larval establishment in phloem. Constitutive and induced
defensive compounds in phloem, including phenolics, may be toxic to feeding larvae (Morewood et al. 2004, Eyles et al. 2007). Wound periderm (i.e. callus tissue) induced by feeding larvae may encapsulate and kill larvae (Anderson 1944, Dunn et al. 1990, Fierke and Stephen 2008).

Previous research indicates that growth of wound periderm induced by larval feeding may be important to birch resistance to bronze birch borer. Wound periderm formation has been associated with resistance to bronze birch borer in paper birch (Miller et al. 1991), but is not associated with resistance in European white birch (B. pendula Roth), which suggests that formation of wound periderm alone is not sufficient to confer resistance (Miller et al. 1991). This is consistent with the hypothesis by Eyles et al. 2007 which postulates that woody angiosperm resistance to wood-borers may result from integration of physical and biochemical defenses. Specifically, induced, targeted defenses selected by coevolution may slow the growth and movement of larvae, allowing wound periderm to encapsulate larvae. In the case of birch and bronze birch borer, the coevolutionary history between paper birch and bronze birch borer may have led to selection of specific defenses that slow bronze birch borer feeding enough to facilitate encapsulation of larvae by wound periderm. Conversely, the lack of targeted defenses in European white birch, which does not share a coevolutionary history, may allow larvae to move more quickly through phloem, thus escaping encapsulation by wound periderm.

In birch species, phenolics, including simple phenolics, tannins, and lignin, are dominant secondary metabolites that have been implicated as defenses in other birch-herbivore interactions (Julkunen-Tiitto et al. 1996, Ossipov et al. 1996, Mämmelä 2001, Kopper et al. 2002, Laitinen et al. 2004, Agrell et al. 2005, Mattson et al. 2005, Tiimonen...
et al. 2005), and therefore may be important to birch resistance to bronze birch borer. However, it has been suggested that nutritive compounds can also act as defenses (Haukioja et al. 1991, Berenbaum 1995) and/or can mitigate or augment the effectiveness of secondary metabolites (Slansky 1992, Simpson and Raubenheimer 2001, Haukioja 2003, Roslin and Salminen 2008, Barbehenn et al. 2009). In particular, concentrations of nitrogen, in the form of free amino acids and proteins, can limit insect growth and reproduction because concentrations are low in plants relative to that required by insects (Southwood 1973, Ayres et al. 2000). Very low plant nitrogen concentrations (or low quality of nitrogenous compounds) can lead to compensatory feeding by insects, resulting in higher intake of defenses, and could reduce suitability of the plant to support insect development.

Previous research identified constitutively expressed phenolics that were present in phloem of resistant paper birch but lacking in more susceptible European white birch, as well as phenolics that were expressed in higher concentrations in paper birch. These differences may contribute to resistance of paper birch against bronze birch borer (Chapter 2). Building off this work, the goal of this experiment was to compare the induced responses in phloem of paper birch to European white birch. Specifically, we compared concentrations of phenolics, tannins, lignin, free amino acids, carbon, and nitrogen as well as growth rate of wound periderm induced by mechanical wounding and by bronze birch borer homogenate. We predicted that paper birch would have faster growth rate of wound periderm and different or higher concentrations of induced defensive chemicals than European white birch. We additionally predicted that paper
birch would have lower nutritive quality (as indicated by decreased concentrations of nitrogen and/or free amino acids) than European white birch.

Methods

Common garden study site and experimental design

Paper birch and European white birch have been planted in a common garden for 13 years at The Ohio State University’s Agricultural Research and Development Center (OARDC) in Wooster, Ohio, USA. The trees are planted in a completely randomized block design and have a 3 m X 3 m spacing. In total, there are 56 paper birch and 54 European white birch. Paper birch and European white birch had mean stem diameters of 11.0 ± 0.3 (SE) and 15.5 ± 0.4 cm, respectively.

The experimental design was a split-plot design with species as the whole plot factor replicated within a block and induction treatment (see following paragraph) as the subplot factor. Four trees of each species were randomly chosen in each of the eight blocks (N=64 trees) to which induction treatments were applied.

Induction treatments

In order to analyze induced responses of birch phloem, induction treatments were applied to the phloem of birch stems on 17 June 2008 (to correspond with natural phenology of bronze birch borer neonate feeding) (Akers and Nielsen 1984). Induction
treatments were applied to three sites on the stem, approximately 1.3 m above ground level. Treatments were arranged to correspond with the points of an equilateral triangle, were applied randomly within each triangular arrangement, and were 10 cm apart. Two sites were wounded using a sterilized 5 mm punch that removed a phloem plug. Twenty-five mg freeze-dried bronze birch borer larval homogenate was inserted into one wound in order to analyze localized induced responses specific to bronze birch borer. The wound without bronze birch homogenate was used to analyze localized induced responses to mechanical damage. Both wounds were covered with a 2 % agar plug, parafilm, and duct tape. The third site remained unwounded in order to quantify constitutive phloem chemistry and to serve as a baseline in order to determine induced responses. Seventy-two hours after wounding, phloem samples were excised from all three treatment locations using a 15 mm punch, which was centered around smaller wounds in order to sample surrounding tissue. Samples were flash frozen in liquid nitrogen and stored at -80°C until they were ground for chemical analysis.

Approximately two months after excising phloem samples, the diameter of each wound was measured using calipers. Growth rate of wound periderm (mm per day) was calculated by subtracting the final diameter of the wound from the original diameter and dividing by the number of days that wound periderm could grow.

Analysis of low molecular weight phenolics

Quantification and identification of phenolics were completed as previously described in chapter 2. In brief, 100 mg of ground phloem tissue was extracted twice in
500 µL of HPLC grade methanol (Fisher, Pittsburgh, PA) for 24 h at 4°C (Bonello and Blodgett 2003). The supernatants were combined and stored in a 1.5-ml microcentrifuge tube at -20°C until analysis by high-performance liquid chromatography (HPLC).

HPLC-MS analysis was completed using a Varian 212-LC chromatography pump, a ProStar 410 HPLC autosampler, a Varian Polaris™ RP18, 4.6 X 150 mm column, and a Varian 500-MS ion trap mass spectrometer operating in the ESI⁺ mode. The MS detector had a capillary voltage of 80 V and a cone voltage of 5000 V. Scans were completed from 100-1400 m/z to measure [M-H] ions.

Quantification of compounds was completed using a Waters 2690 separations module (Milford, MA) equipped with a Waters Xterra™ RP18, 5 µm, 4.6 X 150 mm column, a Waters Xterra™ RP18, 3.9 µm, 3.0 X 20 mm guard column, and a 996 photodiode array detector. The binary mobile phase consisted of 2% glacial acetic acid in HPLC-grade water (solvent A) and 2% glacial acetic acid in HPLC-grade methanol (solvent B). The flow rate was 1 mL/min. The solvent program was as follows (percentages represent proportion of B): 0% (0-2 min); 0-10% (2-4 min); 10-48% (4-20 min); 48-100% (20-38 min); 100% (38-39 min); 100-0% (39-41 min). The injection volume for each sample was 10 uL.

The standards catechin (Alexis Biochemicals, San Diego, CA, USA), chlorogenic acid (MP Biomedicals, Solon, OH, USA), and salicin (Sigma Aldrich, Milwaukee, WI, USA) were used to quantify phenolics. The glycosides catechin pentoside, betuloside, betuloside pentoside, and diarylheptanoids were expressed as salicin equivalents. Catechin and procyanidins were expressed as catechin equivalents. Coumaroylquinic
acid, platyphylloside and unknown peaks 9-12 were expressed as chlorogenic acid equivalents. Concentrations of phenolics were expressed as mg per g fresh weight.

*Analysis of total phenolics, condensed tannins, and lignin*

Concentrations of total phenolics and condensed tannins were analyzed as in Nitao et al. 2001. Briefly, ground phloem samples were extracted with 50% methanol. Extracts were then analyzed using a Rapid Flow Analyzer (Astoria-Pacific, OR), which utilizes a colorimetric analysis method. Total phenolics were analyzed using the Folin-Denis assay. In this assay, samples are diluted with 1.5% sodium lauryl sulfate and mixed with Folin-Denis reagent and sodium carbonate solutions within the Rapid Flow Analyzer. Total phenolics were monitored and analyzed at 580 nm using tannic acid as a standard for quantification. To analyze condensed tannins, 5% concentrated hydrochloric acid in butanol and 2% ammonium sulfate in 2N hydrochloric acid were added to sample extracts. Condensed tannins were monitored and analyzed at 750 nm using quebracho tannin as a standard for quantification. Concentrations of total phenolics and tannins were expressed as mg per g fresh weight.

Concentrations of lignin were analyzed as described by Bonello and Blodgett 2003. This method uses a series of solvents, acids, and bases to remove soluble phenolics and phenolics bound in the cell wall from the lignin in the sample. The lignin was quantified at 280 nm in a spectrophotometer using lignin (Aldrich Chemical Co., Milwaukee, WI, USA) as a standard. Concentrations of lignin were expressed as mg per g fresh weight.
Analysis of carbon, nitrogen, and amino acids

Five mg of ground phloem tissue were used to quantify the C:N. Analysis was completed using a Thermo Electron Corporation (CE Instruments) Flash EA-1112 C-N Analyzer with an AS 3000 Autosampler.

To identify and quantify free amino acids, 25 mg of phloem tissue was extracted in 500 uL 0.01 N hydrochloric acid. Free amino acids in the extract were derivatized using the EZ:faast® GC-MS physiological amino acid analysis kit (Phenomenex Inc., Torrence, CA). GC-MS was completed using an Agilent 6890 Series GC System equipped with a 5973 Mass Selective Detector, a 7683B Series autoinjector and a 7683 Series autosampler. All free amino acids except GABA were identified and quantified using internal and external amino acid standards included in the kit. GABA was identified and quantified using GABA (Sigma, Saint Louis, MO, USA) as an external standard. Concentrations of amino acids were expressed as nmol per mg fresh weight.

Data analysis

Data were analyzed by analysis of variance (ANOVA; PROC GLM) ($\alpha = 0.05$) using SAS 9.1 (SAS Institute. 1999). Following a significant $F$ test, probabilities for differences of the least squared means (PDIFF) were used comparisons among induction treatments and induction treatment by species interactions.

Multivariate analyses were conducted using principal component analysis (PCA) using Minitab 15 (Minitab Inc. 2007) to compare interspecific variation of potential
resistant traits. For analyses that included variables with different units, data were transformed to z-scores prior to analysis.

Results

Wound periderm

Growth rate of wound periderm did not differ between paper birch and European white birch (0.056 ± 0.008 and 0.058 ± 0.008 mm/day, respectively) ($F_{1,55}$=0.14, $P$=0.708). Rate of wound periderm growth over punctures created by sampling phloem around induction treatments were the same between species ($F_{1,124}$=0.85, $P$=0.432). However, in both species, mechanical wounding accelerated subsequent growth over phloem sampling punctures (0.064 ± 0.004 mm/day) compared to rates over phloem punctures following the constitutive treatment (0.047 ± 0.004 mm/day) and bronze birch borer treatment (0.058 ± 0.004 mm/day) ($F_{1,124}$=3.84, $P$=0.024).

Low molecular weight phenolics

Coumaroylquinic acid and diarheptanoid hexoside were detected only in paper birch. Concentrations of betuloside pentoside B, a centrolobol glycoside, centrolobol hexoside, and four unknown phenolics were higher in paper birch (Table 3.1). Concentrations of catechin pentoside, catechin, betuloside pentoside A, betuloside,
Table 3.1. *F*-values from analysis of variance (ANOVA) of concentrations of secondary and primary metabolites in phloem tissues of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*) to induction treatments.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Species</th>
<th>Induction Treatment</th>
<th>Species X Induction Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secondary metabolites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>procyanidin dimer</td>
<td>2.9</td>
<td>7.4***</td>
<td>0.7</td>
</tr>
<tr>
<td>catechin pentoside</td>
<td>59.0****</td>
<td>11.0****</td>
<td>1.5</td>
</tr>
<tr>
<td>catechin</td>
<td>14.25***</td>
<td>7.8***</td>
<td>1.1</td>
</tr>
<tr>
<td>procyanidin trimer</td>
<td>5.6*</td>
<td>5.4**</td>
<td>0.9</td>
</tr>
<tr>
<td>coumaroylquinic acid</td>
<td>paper birch only</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>betuloside pentoside A</td>
<td>13.2***</td>
<td>4.7**</td>
<td>2.8</td>
</tr>
<tr>
<td>betuloside</td>
<td>8.0**</td>
<td>5.4**</td>
<td>2.3</td>
</tr>
<tr>
<td>betuloside pentoside B</td>
<td>17.7****</td>
<td>4.9**</td>
<td>1.0</td>
</tr>
<tr>
<td>unknown 1</td>
<td>14.7***</td>
<td>10.3****</td>
<td>1.7</td>
</tr>
<tr>
<td>unknown 2</td>
<td>47.7****</td>
<td>5.5**</td>
<td>0.2</td>
</tr>
<tr>
<td>unknown 3</td>
<td>42.8****</td>
<td>7.4***</td>
<td>0.8</td>
</tr>
<tr>
<td>unknown 4</td>
<td>10.0**</td>
<td>4.0*</td>
<td>4.6*</td>
</tr>
<tr>
<td>platyphylloside</td>
<td>72.6****</td>
<td>4.3**</td>
<td>2.3</td>
</tr>
<tr>
<td>diarylheptanoid hexoside</td>
<td>paper birch only</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>centrolobol glycoside 1</td>
<td>12.0***</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>centrolobol glycoside 2</td>
<td>33.1****</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>centrolobol hexoside</td>
<td>4.4*</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>total phenolics</td>
<td>111.2****</td>
<td>8.87***</td>
<td>0.85</td>
</tr>
<tr>
<td>tannins</td>
<td>84.7****</td>
<td>9.00</td>
<td>0.6</td>
</tr>
<tr>
<td>lignin</td>
<td>0.46</td>
<td>5.55**</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Primary metabolites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alanine</td>
<td>13.1***</td>
<td>44.4****</td>
<td>4.4**</td>
</tr>
<tr>
<td>glycine</td>
<td>0.0</td>
<td>30.3****</td>
<td>1.7</td>
</tr>
<tr>
<td>valine</td>
<td>8.7**</td>
<td>16.8****</td>
<td>0.7</td>
</tr>
<tr>
<td>serine</td>
<td>11.7***</td>
<td>5.4**</td>
<td>2.3</td>
</tr>
<tr>
<td>GABA</td>
<td>11.7***</td>
<td>6.1**</td>
<td>2.3</td>
</tr>
<tr>
<td>proline</td>
<td>0.1</td>
<td>15.5****</td>
<td>0.7</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>1.6</td>
<td>61.2****</td>
<td>4.2*</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>0.01</td>
<td>3.1*</td>
<td>4.5**</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>0.3</td>
<td>37.2****</td>
<td>3.3*</td>
</tr>
<tr>
<td>histidine</td>
<td>10.3**</td>
<td>45.1****</td>
<td>5.8**</td>
</tr>
<tr>
<td>lysine</td>
<td>10.9**</td>
<td>50.8****</td>
<td>1.6</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.06</td>
<td>5.9**</td>
<td>0.0</td>
</tr>
<tr>
<td>tryptophan</td>
<td>1.5</td>
<td>27.4****</td>
<td>1.1</td>
</tr>
<tr>
<td>essential amino acids</td>
<td>19.5***</td>
<td>54.5****</td>
<td>3.63*</td>
</tr>
<tr>
<td>total amino acids</td>
<td>4.01*</td>
<td>13.4****</td>
<td>3.31*</td>
</tr>
<tr>
<td>nitrogen</td>
<td>11.7***</td>
<td>39.7****</td>
<td>2.15</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Bold indicates compounds that have significantly higher concentrations or only detected in paper birch.

GABA, gamma-aminobutyric acid.
platyphyllloside and a centrolobol glycoside were higher in European white birch (Table 3.1).

Individual phenolics of paper birch and European white birch responded similarly to induction treatments, as interactions between induction treatments and species were not observed (Table 3.1). Mechanical wounding and bronze birch borer homogenate decreased concentrations of one phenolic (unknown phenolic 4) in European white birch but not in paper birch (Table 3.2).

Induction treatments affected concentrations of individual phenolics. Mechanical wounding and bronze birch borer homogenate decreased concentrations of procyanidin dimer, catechin pentoside, catechin, betuloside, betuloside pentoside B, and unknown phenolics 1, 2, and 3.

**Total phenolics, condensed tannins, and lignin**

European white birch had higher concentrations of Folin-Denis total phenolics and condensed tannins than paper birch (Table 3.1). There was no difference in concentrations of lignin between species. Interactions between induction treatments and species did not affect concentrations of total phenolics, tannins, or lignin between paper birch and European white birch (Fig. 3.1).

Induction treatments affected concentrations of tannins and lignin but not Folin-Denis total phenolics (Table 3.1). Bronze birch borer homogenate suppressed concentrations of tannins. Mechanical wounding and bronze birch borer homogenate increased concentrations of lignin.
Table 3.2. Constitutive and induced concentrations of secondary metabolites (mg/g fresh weight ± SE) in phloem tissues of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>European white birch</th>
<th>Paper birch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Constitutive control</td>
<td>Mechanical wound</td>
</tr>
<tr>
<td></td>
<td>Mechanical wound +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bronze birch borer</td>
<td></td>
</tr>
<tr>
<td>Procyanidin dimer</td>
<td>13.07 ± 0.58 a</td>
<td>9.74 ± 0.57 b</td>
</tr>
<tr>
<td>Catechin pentoside</td>
<td>27.60 ± 0.73 a</td>
<td>13.89 ± 0.72 c</td>
</tr>
<tr>
<td>Catechin</td>
<td>14.27 ± 0.58 a</td>
<td>9.30 ± 0.57 c</td>
</tr>
<tr>
<td>Procyanidin trimer</td>
<td>12.86 ± 0.22 a</td>
<td>9.75 ± 0.22 a</td>
</tr>
<tr>
<td>Coumaroylquinic acid</td>
<td>0.00 ± 0.00 b</td>
<td>3.20 ± 0.09 a</td>
</tr>
<tr>
<td>Betulose pentoside A</td>
<td>11.90 ± 0.53 a</td>
<td>5.75 ± 0.52 c</td>
</tr>
<tr>
<td>Betulose</td>
<td>12.54 ± 0.54 a</td>
<td>8.06 ± 0.53 c</td>
</tr>
<tr>
<td>Betulose pentoside B</td>
<td>3.79 ± 0.47 a</td>
<td>7.15 ± 0.47 c</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>7.95 ± 0.56 b</td>
<td>12.15 ± 0.55 a</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>2.21 ± 0.33 b</td>
<td>6.85 ± 0.32 a</td>
</tr>
<tr>
<td>Unknown 3</td>
<td>4.86 ± 0.54 b</td>
<td>9.35 ± 0.53 a</td>
</tr>
<tr>
<td>Unknown 4</td>
<td>4.88 ± 0.52 b</td>
<td>5.98 ± 0.51 a</td>
</tr>
<tr>
<td>Platiphyllloside</td>
<td>34.92 ± 0.89 a</td>
<td>24.32 ± 0.88 c</td>
</tr>
<tr>
<td>Diaryleptanoid hexoside</td>
<td>0.00 ± 0.21 a</td>
<td>8.14 ± 0.21 a</td>
</tr>
<tr>
<td>Centroleobol glycoside</td>
<td>1.20 ± 0.09 b</td>
<td>2.04 ± 0.15 ab</td>
</tr>
<tr>
<td>Centroleobol glycoside</td>
<td>3.51 ± 0.14 a</td>
<td>2.36 ± 0.13 b</td>
</tr>
<tr>
<td>Centroleobol hexoside</td>
<td>1.52 ± 0.15 c</td>
<td>2.04 ± 0.15 ab</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>89.03 ± 2.13 a</td>
<td>72.04 ± 2.17 c</td>
</tr>
<tr>
<td>Tannins</td>
<td>97.46 ± 4.13 a</td>
<td>64.58 ± 4.21 b</td>
</tr>
<tr>
<td>Lignin</td>
<td>1.34 ± 0.07 a</td>
<td>1.43 ± 0.07 a</td>
</tr>
</tbody>
</table>

Means within a row followed by different letters are significantly different using PDIF (α = 0.05).

Standard errors given are the pooled standard errors associated with PDIF among species by induction treatment combinations.
Figure 3.1. Constitutive and induced concentrations of A total phenolics, B tannins, and C lignin in phloem of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*). Probabilities for differences of the least squared means (PDIFF) from analysis of variance (ANOVA) were used to test for differences. Standard errors shown are the pooled standard errors associated with PDIFF among species by induction treatment combinations. Bars with different letters are significantly different ($\alpha = 0.05$).
Carbon, nitrogen, and amino acids

European white birch had higher concentrations of nitrogen (Table 3.1) and carbon than paper birch. Bronze birch borer homogenate increased concentrations of carbon and nitrogen in paper birch and European white birch.

European white birch had higher concentrations of total amino acids, essential amino acids, and five individual amino acids (alanine, GABA, lysine, serine, and valine) than paper birch (Table 3.1). Induction treatments affected concentrations of total amino acids, essential amino acids, and five individual amino acids (alanine, aspartic acid, glutamic acid and phenylalanine) in paper birch and European white birch (Table 3.1, Fig. 3.2). Mechanical wounding and/or bronze birch borer homogenate decreased concentrations of total amino acids, essential amino acids, aspartic acid, glutamic acid, and phenylalanine in paper birch and European white birch. Mechanical wounding and bronze birch borer inoculation increased concentrations of alanine in paper birch and European white birch (Table 3.3).

Multivariate response of potential resistant traits

Principal component analysis of traits induced by bronze birch borer homogenate showed separation between species along the diagonal between the first and second PC axis, which together explained 18.6 % of the total variation (Fig. 3.3A). Location along the first PC axis was primarily determined by the amino acids valine (EV: 0.25), leucine
Figure 3.2. Constitutive and induced concentrations of A total amino acids and B essential amino acids in phloem of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*). Probabilities for differences of the least squared means (PDIFF) from analysis of variance (ANOVA) were used to test for differences. Standard errors shown are the pooled standard errors associated with PDIFF among species by induction treatment combinations. Bars with different letters are significantly different ($\alpha = 0.05$).

![Graph A](image1)

**A**

<table>
<thead>
<tr>
<th>Species</th>
<th>Induction treatment</th>
<th>Species X Induction treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.047</td>
<td></td>
<td>0.040</td>
</tr>
</tbody>
</table>

![Graph B](image2)

**B**

<table>
<thead>
<tr>
<th>Species</th>
<th>Induction treatment</th>
<th>Species X Induction treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 0.001</td>
<td></td>
<td>0.029</td>
</tr>
</tbody>
</table>
Table 3.3. Constitutive and induced concentrations (nmol/mg fresh weight ± SE) of free amino acids in phloem tissues of European white birch (*Betula pendula*) and paper birch (*Betula papyrifera*).

<table>
<thead>
<tr>
<th>Primary metabolites</th>
<th>European white birch</th>
<th>Paper birch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Constitutive control</td>
<td>Mechanical wound</td>
</tr>
<tr>
<td>alanine</td>
<td>0.18 ± 0.01 c</td>
<td>0.22 ± 0.01 b</td>
</tr>
<tr>
<td>glycine</td>
<td>0.06 ± 0.00 a</td>
<td>0.04 ± 0.00 b</td>
</tr>
<tr>
<td>valine</td>
<td>0.05 ± 0.00 b</td>
<td>0.05 ± 0.00 ab</td>
</tr>
<tr>
<td>serine</td>
<td>0.32 ± 0.01 b</td>
<td>0.35 ± 0.01 b</td>
</tr>
<tr>
<td>GABA</td>
<td>0.18 ± 0.01 b</td>
<td>0.19 ± 0.01 b</td>
</tr>
<tr>
<td>proline</td>
<td>0.04 ± 0.00 a</td>
<td>0.03 ± 0.00 b</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>1.38 ± 0.04 a</td>
<td>0.92 ± 0.04 b</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>1.37 ± 0.04 a</td>
<td>1.22 ± 0.04 b</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>0.02 ± 0.00 b</td>
<td>0.03 ± 0.00 a</td>
</tr>
<tr>
<td>histidine</td>
<td>0.22 ± 0.01 a</td>
<td>0.07 ± 0.01 b</td>
</tr>
<tr>
<td>lysine</td>
<td>0.12 ± 0.01 a</td>
<td>0.06 ± 0.01 c</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.07 ± 0.01 a</td>
<td>0.02 ± 0.00 b</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.08 ± 0.01 a</td>
<td>0.04 ± 0.01 bc</td>
</tr>
<tr>
<td>essential amino acids</td>
<td>0.53 ± 0.02 a</td>
<td>0.29 ± 0.02 b</td>
</tr>
<tr>
<td>total amino acids</td>
<td>4.32 ± 0.12 a</td>
<td>3.63 ± 0.12 c</td>
</tr>
</tbody>
</table>

Means within a row followed by different letters are significantly different using PDIF from ANOVA (α = 0.05).

Standard errors given are the pooled standard errors associated with PDIF among species by induction treatment combinations.

GABA, gamma-aminobutyric acid.
Figure 3.3. Variation in A induced phytochemistry to bronze birch borer (*Agrilus anxius*) and B constitutive phytochemistry between paper birch (*Betula papyrifera*) and European white birch (*Betula pendula*), and variation in C free amino acids among induction treatments in both species. Percentage of total variability explained by the first two axes is shown on each analysis.

continued
Fig. 3.3 continued
(EV: 0.23), serine (EV: 0.25), GABA (EV: 0.26), and aspartic acid (EV: 0.25). Location along the second PC axis was primarily determined by the phenolics catechin pentoside (EV: 0.27), catechin (EV: 0.21), platyphylloside (EV: 0.25), total phenolics (EV: 0.29) and tannins (EV: 0.28), which were associated with European white birch, and coumaroylquinic acid (EV: -0.22) and diarylheptanoid hexoside (EV: -0.23), which were associated with paper birch.

Principal component analysis of constitutively expressed phytochemicals showed separation between species along the diagonal between the first and second PC axis, which together explain 16.97% of the total variation (Fig. 3.3B). Location along the first PC axis was primarily determined by the amino acids alanine (EV: 0.27), valine (EV: 0.28), leucine (EV: 0.28), and proline (EV: 0.28). Location along the second PC axis was determined by the catechin pentoside (EV: 0.31), catechin (EV: 0.28), platyphylloside (EV: 0.29), tannins (EV: 0.30), and total phenolics (EV: 0.31), which were associated with European white birch.

Principal component analysis of free amino acids showed separation between constitutive and induced wounding treatments along the second PC axis, which explained 16.0% of the variation (Fig. 3.3C). The first two principal components explained 62.9% of the total variation. Location along the first PC axis was determined by the amino acids valine (EV: 0.316), GABA (EV: 0.306), and proline (EV: 0.325). Location along the second PC axis was determined primarily by lysine (EV: -0.380), histidine (EV: -0.429), tyrosine (EV: -0.383) and tryptophan (EV: -0.447), which were associated with the constitutive treatment. Species could not be distinguished from one another by principal
Discussion

In previous research, birch resistance to bronze birch borer was related to wound periderm formation in paper birch but not in European white birch (Miller et al. 1991). In this study, there was no difference between rates of wound periderm formation in paper birch and European white birch. However, wound periderm may contribute to birch resistance if it operates by one of the following mechanisms. First, the composition of wound periderm may be different between species, leading to effective encapsulation of larvae in resistant species but not the more susceptible species. Alternatively, wound periderm may work in tandem with another plant attribute, such as a defensive metabolite that is not part of wound periderm. For example, in resistant species, presence of specific defenses that have been selected for during coevolution with bronze birch borer, which are lacking in susceptible species, may slow the movement of the larva enough to allow wound periderm to isolate, encapsulate, and ultimately kill the larva (Biggs et al. 1984, Eyles et al. 2007).

The induced chemical profiles of paper birch and European white birch phloem were different (Fig. 3A), predominantly due to qualitative and quantitative differences in phenolic composition. Similarly, the constitutive chemical profiles of paper birch and European white birch were also distinct (Fig. 3B), and differences were largely due to variation in phenolic composition, although differences in concentrations of free amino
acids may also contribute to separation between species. Induced and constitutive
defensive profiles of paper birch contained coumaroylquinic acid and diarylheptanoid
hexoside, which were not detected in European white birch. Induced and constitutive
concentrations of seven additional phenolics were higher in paper birch than in European
white birch, and six of these (betuloside pentoside B, a centrolobol glycoside and 4
unidentified compounds) were also in higher constitutive concentrations in paper birch
than in European white birch in previous research (Chapter 2). This might indicate that
interspecific differences in concentrations of these phenolics are genetically regulated and
are robust to changes that can be associated with year-to-year environmental variation.

Concentrations of total phenolics, condensed tannins, and free amino acids also
differed between species. European white birch, which is more susceptible to bronze
birch borer, had higher concentrations of induced and constitutive condensed tannins and
total phenolics than more resistant paper birch. This indicates that total pools of phenolics
and tannins do not confer resistance to birch against bronze birch borer. However, these
finding do not preclude the possibility that individual phenolics or tannins could be
important to resistance of paper birch to bronze birch borer. For example, although
concentrations of total phenolics were higher in more susceptible European white birch,
concentrations of several individual phenolics were higher in paper birch and may be
important to resistance. This emphasizes the importance of measuring individual
compounds when studying plant-insect interactions, rather than pools of metabolites
alone, which cannot capture differences in the quality of metabolic pools (Moilanen and
Salminen 2008). Additionally, European white birch, which is susceptible to bronze birch
borer, had higher induced and constitutive concentrations of total and essential free
amino acids in phloem tissues than paper birch. Free amino acids are a small component of the total nitrogen present in plant tissue. However, they can be readily metabolized whereas complex proteins require catabolic processing before utilization (Cockfield, 1988), and therefore small differences in absolute amounts might impact insect performance.

Experimentally applied bronze birch borer homogenate in combination with mechanical wounding did not elicit phytochemical responses that were different from those induced by mechanical wounding alone. In pine stems, similar experimental techniques of inoculation with bark beetles and/or associated fungi are effective surrogates for natural herbivory and subsequent fungal inoculation, and are capable of inducing specific localized host responses, such as increased production of individual monoterpenes (Raffa and Berryman 1982, 1983, Klepzig et al. 1995). Similarly, in leaves, proteins and other bioelicitors associated with insect oral secretions induce specific plant responses, including induction of reactive oxygen species, plant hormones, defensive proteins, and secondary metabolites (Baldwin et al. 2001, Major and Constabel 2006, Wu and Baldwin 2009, Bricchi et al. 2010). In our study, mechanical wounding of stem phloem (with or without bronze birch borer homogenate) induced responses in measured phytochemicals; however, it is not known if the responses observed simulate the natural responses of paper birch and European white birch to oviposition by adult bronze birch borer females and/or feeding by bronze birch borer larvae.

Differences between induced and constitutive profiles in phloem of paper birch and European white birch were largely driven by quantitative variation in concentrations of amino acids (Fig. 3C), although concentrations of phenolics and lignin also changed.
In both species, concentrations of free amino acids decreased following wounding whereas concentrations of nitrogen increased. These changes may indicate an increased demand for enzymes and other proteins necessary to synthesize tissues involved in wound healing. Concentrations of total phenolics and many individual phenolics also decreased following wounding in both species, which may indicate that they are utilized to make more complex polyphenolics such as lignin. Concentrations of lignin increased in both species only 72 h following wounding, whereas in cypress phloem, increases in lignin deposits were detected by microscopy seven days following wounding (Kusumoto 2005). Rapid increases in lignin concentrations are likely part of the early formation of wound periderm (callus) tissue (Walter and Schadel 1983, Biggs 1985). Although deposition of lignin is part of a general response to wounding, increases in lignin could also serve a defensive function both physically, by increasing plant toughness, and physiologically, by reducing digestibility of cell wall carbohydrates like cellulose and hemicellulose (Hagerman and Butler 1991).

In summary, paper birch and European white birch have distinct induced and constitutive chemical profiles, and variation between species was largely due to qualitative and quantitative differences in phenolic composition. These differences may correspond with the vast variation in resistance of paper birch and European white birch to bronze birch borer. Plant defense is generally complex, and we predict, that as in many other plant-insect interactions, the nature of birch defense to bronze birch borer will not be reduced to the presence of one chemical. Likely many plant traits, including some analyzed in this study, contribute to birch resistance.
Acknowledgements

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References


mandshurica) and two north american ash species (Fraxinus americana and Fraxinus pennsylvanica). J. Chem. Ecol. 33: 1430-1448.


Minitab Inc. 2007. Minitab 15 statistical software, State College, PA.


Chapter 4

Effects of experimental girdling on paper birch defenses, source-sink interactions, and bronze birch borer colonization

Abstract

Outbreaks of bronze birch borer (*Agrilus anxius*), a wood-boring beetle endemic to North America, have historically been associated with stressed hosts (*Betula* spp.). However, the physiological and biochemical mechanisms underlying the shift in host resistance, from a resistant to more susceptible state, are poorly understood. To analyze how stress modulates the relationship between whole plant physiology and tree defenses, paper birch (*B. papyrifera*) stems were girdled, disrupting transport of current photosynthate and altering within-tree resistance to bronze birch borer. Girdling increased susceptibility of paper birch to bronze birch borer colonization, and phloem below the girdle was more susceptible than above the girdle. Girdling reduced concentrations of total phenolics and rate of wound periderm formation below the girdle in 1-year girdled trees. Girdling also reduced concentrations of induced total phenolics below the girdle over time, indicating increased carbon stress as phloem below the girdle continued to be isolated from the flux of current photosynthate. Levels of bronze birch borer colonization were inversely related to levels of induced phenolics below the girdle, but there was no relationship above the
girdle. Our findings confirm that stress compromises resistance of paper birch to bronze birch borer colonization and that the flux of current photosynthate from leaves to the trunk may be an important physiological mechanism underlying paper birch resistance. These results may also suggest that in interactions between coevolved trees and wood-boring insects, maintenance of tree vigor is important in bottom-up regulation of populations of wood-boring insects.
Introduction

Outbreaks of wood-boring insects have long been associated with stressed host trees, frequently in “dieback” or “decline” conditions (Balch and Prebble 1940, Anderson 1944, Dunn et al. 1986, Stephen et al. 2001). Typically wood-boring insects are labeled as secondary pests, meaning they are not able to successfully colonize hosts with which they have coevolved unless a stressor such as drought, physical injury, high temperatures, defoliation, or advanced age compromise host resistance (Balch and Prebble 1940, Anderson 1944, Dunbar and Stephens 1975, Cote and Allen 1980, Jones et al. 1993, Haavik et al. 2008). However, the physiological and biochemical mechanisms underlying the shift in host resistance, from a resistant to a more susceptible state, are not understood.

Bronze birch borer (*Agrilus anxius* Gory) (Coleoptera: Buprestidae), a wood-boring beetle endemic to North America, colonizes *Betula* spp. and is prone to periodic, widespread outbreaks (Balch and Prebble 1940, Jones et al. 1993). Paper birch (*B. papyrifera* Marshall) shares a coevolutionary history with bronze birch borer and is resistant (Nielsen et al. *in press*) unless stressed. Increased susceptibility has been associated with drought (Jones et al. 1993), experimental girdling (Anderson 1944, Barter 1957), high temperatures (Jones et al. 1993), and advanced age (Balch and Prebble 1940). Bronze birch borer larvae feed in stem phloem tissue, producing galleries (Barter 1957). Galleries disrupt translocation of photosynthate, which can girdle and eventually kill trees (Barter 1957).
Investigations of interactions between woody-angiosperms and wood-boring insects have led to several proposed mechanisms of tree resistance, which may co-occur and work interactively (Eyles et al. 2007). Physical and chemical mechanisms that are constitutively present and/or induced by larval feeding may contribute to resistance. Physical mechanisms include high constitutive bark moisture (Hanks et al. 1991, Hanks et al. 1999) and sap flow (Morewood et al. 2004), which interfere with larval establishment. Wound periderm formation (i.e. callus tissue) induced by larval feeding may physically encapsulate and crush feeding larvae (Anderson 1944, Dunn et al. 1990, Fierke and Stephen 2008). Constitutive and induced defensive chemicals, including phenolics, may be toxic or deterrent to herbivores (Morewood et al. 2004, Eyles et al. 2007).

Girdling, which is the removal of a band of phloem and bark tissue from the cambium around the trunk of a tree, alters tree resistance to herbivores by disrupting connectivity among sources and sinks within the tree (Anderson 1944, Christiansen and Ericsson 1986). Girdling obstructs phloem transport of current photosynthate from the leaves to portions of the tree below the girdle, resulting in accumulation of photosynthate above the girdle and a depletion of photosynthate below the girdle (Noel 1970, Goren et al. 2004). The transport of water and minerals in the xylem is less affected (Noel 1970). In conifers, the defensive capacity of stems against bark beetles and associated fungi comes from both local reserves and long-distance transport of carbohydrates in the phloem (Christiansen and Ericsson 1986, Arnold and Schultz 2002, Bonello et al. 2006). Because local reserves can be rapidly depleted, tree resistance becomes dependent on the ability to mobilize carbohydrates needed for biosynthesis of induced defenses at the site.
of damage or infestation (Christiansen and Ericsson 1986, Christiansen et al. 1987). Thus, as tissues below a girdle are isolated from long-distance translocation of carbohydrates from sources above the girdle, they may exhibit a reduced capacity to induce defenses as local reserves are diminished.

In woody angiosperms, girdling decreases host resistance for Agrilus spp. as beetles preferentially colonize experimentally girdled trees (Dunn et al. 1986, Dunn et al. 1987, Crook et al. 2008). In paper birch specifically, girdling increases susceptibility to bronze birch borer (Anderson 1944, Barter 1957), and within a tree, stem phloem below the girdle is more suitable for bronze birch borer larval development than stem phloem above the girdle (Anderson 1944). This within-tree variability in host resistance facilitates analysis of resistance without confounding variation from differences in genotype and the environment. Therefore, to analyze how stress modulates the relationship between whole plant physiology and defenses, paper birch stems were girdled, disrupting transport of current photosynthate and altering within-tree resistance to bronze birch borer. We hypothesized that paper birch resistance to bronze birch borer relies on current photosynthate that is translocated from the leaves to the trunk and, therefore, that the section of phloem above the girdle will be more resistant to bronze birch borer, and the section of phloem below the girdle will be more susceptible. However, we hypothesized that resistance of girdled trees, both above and below the girdle, will decrease over time as source strength weakens. Specifically, we compared rate of wound periderm growth, constitutive and induced defensive chemistry, and bronze birch borer colonization above and below the girdle in paper birch over time.
Methods

Study site and experimental design

This experiment was conducted within a forest located at the U.S. Forest Service Northern Research Station near Rhinelander, WI, USA. Thirty paper birch were randomly selected, which had mean stem diameters of $10.02 \pm 0.60$ (SE) cm at 1.5 m above ground level. Trees were blocked into trios based on diameter and proximity. Within each trio, trees were randomly assigned one of three treatments: 1-year girdle, 2-year girdle, and ungirdled control. After girdling treatments were applied, three induction treatments above and below the girdle were also applied.

Girdling treatments

Ten paper birch were girdled on 11 June 2007 (2-year girdle), ten additional paper birch were girdled on 29 May 2008 (1-year girdle), and ten paper birch were left ungirdled (control). Using a handsaw and hatchet, trees were girdled by removing approximately a 10 cm band of phloem, approximately 1.5 m above ground level.

Induction treatments

In order to analyze constitutive and induced resistance mechanisms of paper birch, three induction treatments were applied above and below the girdle to the phloem.
of birch stems. Induction treatments were applied on 9 July 2008 to correspond with the phenology of bronze birch borer neonate feeding under natural conditions (Akers and Nielsen 1990). Treatments above the girdle were approximately 1.8 m above ground level and treatments below the girdle were approximately 0.9 m above ground level. Treatments were arranged to correspond with the points of an equilateral triangle, were applied randomly within each triangular arrangement, and were 10 cm apart. Two of the three treatments were administered using a 5 mm punch that removed a phloem plug. Twenty-five mg of freeze-dried bronze birch borer larval homogenate was inserted into one wound in order to analyze localized induced responses specific to bronze birch borer. The wound without bronze birch homogenate was used to analyze localized induced responses to mechanical damage. Both wounds were covered with a 2% agar plug, parafilm, and duct tape. The third treatment location remained unwounded in order to quantify constitutive phloem chemistry and to serve as a baseline in order to determine induced responses. Seventy-two hours after wounding, phloem samples were excised from all treatment locations, above and below the girdle, using a 15 mm punch that was centered around smaller wounds in order to sample surrounding tissue. Samples were flash frozen in liquid nitrogen and stored at -80°C until they were ground for chemical analysis.

On 17 June 2008, the diameter of each wound was measured using calipers. Growth rate of wound periderm (mm per day) was calculated by subtracting the final diameter of the wound from the original diameter and dividing by the number of days that wound periderm could grow.
Identification and quantification of phenolics were completed as described in chapter 2. Briefly, 100 mg of ground phloem tissue was extracted twice in 500 µL of HPLC grade methanol (Fisher, Pittsburgh, PA) for 24 h at 4°C (Bonello and Blodgett 2003). The supernatants were combined and stored at -20°C until analysis by high-performance liquid chromatography (HPLC).

Identification of compounds was completed using a Varian 212-LC chromatography pump, a ProStar 410 HPLC autosampler, a Varian Polaris™ RP18, 4.6 X 150 mm column, and a Varian 500-MS ion trap mass spectrometer operating in the ESI mode. Capillary voltage on the MS detector was 80 V and cone voltage was 5000 V. Scans were completed from 100-1400 m/z to measure [M-H] ions.

Quantification of compounds was completed using a Waters 2690 separations module (Milford, MA), a Waters Xterra™ RP18, 5 µm, a 4.6 X 150 mm column, a Waters Xterra™ RP18, a 3.9 µm, 3.0 X 20 mm guard column, and a 996 photodiode array detector. The binary mobile phase consisted of 2% glacial acetic acid in HPLC-grade water and 2% glacial acetic acid in HPLC-grade methanol. The solvent program was as follows (percentages represent proportion of methanol): 0% (0-2 min); 0-10% (2-4 min); 10-48% (4-20 min); 48-100% (20-38 min); 100% (38-39 min); 100-0% (39-41 min). The flow rate was 1 mL/min, and the injection volume for each sample was 10 µL.

The standards catechin (Alexis Biochemicals, San Diego, CA, USA), chlorogenic acid (MP Biomedicals, Solon, OH, USA), and salicin (Sigma Aldrich, Milwaukee, WI, USA) were used to quantify phenolics. The glycosides catechin pentoside, betuloside,
betuloside pentoside, and diarylheptanoids were expressed as salicin equivalents. Catechin and procyanidins were expressed as catechin equivalents. Coumaroylquinic acid, platyphylloside and unknown peaks 9-12 were expressed as chlorogenic acid equivalents. Concentrations of phenolics were expressed as mg per g fresh weight.

**Bronze birch borer colonization**

Trees were felled on 12 May 2009. A 1 m log above and below the girdle was collected. The outer bark from the logs was removed using a drawknife until the larval galleries were revealed. The density of larval galleries was estimated using a 10 X 10 cm grid where each square was 1 cm², and colonization was considered positive if a larval gallery intersected a square. The density was estimated at six standardized positions on the logs. These positions included 15, 45, and 75 cm from the base of the north side of the log and 25, 55, and 85 cm on the south side of the log. These data were used to calculate an average estimate of density per log.

**Statistical model and analysis**

This experimental design for analysis of phenolics and wound periderm was a split-split plot design where girdling treatment (1-year girdle, 2-year girdle, and ungirdled control) was the whole plot factor, location relative to girdle (above vs. below) was the split plot factor, and the induction treatment was the split-split plot factor. The experimental design for analysis of bronze birch borer was a split plot design where
girdling treatment was the whole plot factor and location relative to girdle was the split plot factor.

The statistical model for the split-split plot design was

\[ Y_{ijkl} = \mu + B_i + G_j + BG_{ij} + L_k + GL_{jk} + I_l + GI_{jl} + LI_{kl} + GLI_{jkl} + \varepsilon_{ijkl} \]

where \( Y_{ijkl} \) was the response of block \( i \), girdling treatment \( j \), location \( k \), and induction treatment \( l \). Factors including block \( (B_i) \) were random effects. All other factors were considered fixed which included girdling treatment \( (G_j) \), location \( (L_k) \), induction treatment \( (I_l) \) and their interactions. The interaction between girdling treatment and block \( (BG_{ij}) \) was the error for the whole plot unit. Block interactions with location and location by girdling interaction were combined to form the split plot error term \( (B_i(GL_{jk})) \). The residual \( (\varepsilon_{ijkl}) \) was the error term for the split-split plot unit.

Data were analyzed by ANOVA \((\alpha = 0.05)\) using SAS 9.1 (SAS Institute. 1999). One phenolic (centrolobol glycoside 1) did not meet the assumption of normality and was log-transformed to meet this assumption. Following a significant \( F \) test, probabilities for differences of the least squared means (PDIFF) were used for comparisons among girdling by location interactions, induction treatments, girdling by induction treatment interactions, location by induction treatment interactions, and girdling by location by induction treatment interactions. Multivariate analyses were conducted using principal component analysis (PCA) using Minitab 15 (Minitab Inc. 2007). Pearson’s correlation coefficients were used to quantify the relationship between bronze birch borer colonization and individual and total phenolics.
Results

Bronze birch borer colonization

More bronze birch borer colonized birch phloem below versus above the girdle ($F_{1, 27}=9.35, P=0.005$). Additionally, more bronze birch borer colonized 2-year girdled trees (34% of squares with galleries), than 1-year girdled trees (21%) and ungirdled trees (2%) ($F_{2, 18}=15.30, P=0.0001$). Girdling treatment affected levels of bronze birch borer colonization above versus below the girdle ($F_{2, 27}=4.07, P=0.029$) (Fig. 4.1). More bronze birch borer colonized birch below versus above the girdle in 2-year girdled trees. However, there were no differences in levels of colonization above versus below the girdle in ungirdled control trees or 1-year girdled trees (Fig. 4.1).

Wound periderm

Wound periderm formation was faster above the girdle than below ($F_{1, 27}=14.23, P=0.001$). Wound periderm formation was faster in 1-year girdled trees (1 mm growth), than the 2-year girdled trees (0.2 mm growth) or control trees (0 mm growth) ($F_{2, 18}=6.42, P=0.009$). There was no interaction between girdling treatment and location (above versus below the girdle) affecting the rate of wound periderm formation ($F_{2, 27}=2.39, P=0.111$).
Figure 4.1. Levels of bronze birch borer (*Agrilus anxius*) colonization above and below girdling treatments in paper birch (*Betula papyrifera*). Colonization was estimated counting galleries that intersected squares in a 10 X 10 cm grid that was placed six times on birch logs. Probabilities for differences of the least squared means (PDIFF) from analysis of variance (ANOVA) were used for statistical comparisons. Bars with different letters are significantly different ($\alpha = 0.05$).
Phenolics

Concentrations of total phenolics and most individual phenolics were not different above versus below the girdle (Table 4.1). Concentrations of two phenolics, catechin pentoside and platyphylloside, were higher below than above the girdle ($P=0.03; 0.004$, respectively). Girdling treatments did not affect the concentrations of total or individual phenolics (Table 4.1).

Constitutive concentrations of total phenolics, coumaroylquinic acid, an unknown phenolic, and centrolobol glycoside 2 were higher than concentrations induced by bronze birch borer homogenate ($P=0.003; 0.008; 0.007; 0.010$, respectively). Location (above versus below girdle) did not affect the concentrations of total or individual phenolics among induction treatments (Table 4.1).

An interaction between girdling treatment and location affected concentrations of total phenolics and five individual phenolics (catechin pentoside, procyanidin trimer, betulloside pentoside B, centrolobol glycoside 1, centrolobol hexoside) (Table 4.1). In 1-year girdled trees, concentrations of total phenolics, catechin pentoside, betulloside pentoside B, and centrolobol glycoside 1 were higher below versus above the girdle ($P=0.006; 0.001; 0.007; 0.033; 0.005$, respectively), but there were no differences in concentrations above versus below the girdle in 2-year girdled trees ($P=0.216; 0.559; 0.324; 0.106; 0.747$, respectively) or control trees ($P=0.466; 0.489; 0.613; 0.725; 0.922$, respectively). In 1-year girdled trees, concentrations of procyanidin trimer were higher below than above the girdle ($P=0.001$), but in 2-year girdled trees, concentrations of procyanidin trimer were higher above than below the girdle ($P=0.004$). In ungirdled trees,
Table 4.1. *F*-values from analysis of variance (ANOVA) of responses of phenolics in phloem of paper birch (*Betula papyrifera*) to girdling and induction treatments. The girdle treatment (1-year girdle, 2-year girdle, ungirdled control) was the whole plot factor, location relative to girdle (above vs. below) was the split plot factor, and the induction treatment (mechanical wound, bronze birch borer + mechanical wound, unwounded control) was the split-split plot factor.

<table>
<thead>
<tr>
<th>Phenolic</th>
<th>Girdle</th>
<th>Location</th>
<th>Induction Treatment</th>
<th>Girdle X Location</th>
<th>Girdle X Induction Treatment</th>
<th>Location X Induction Treatment</th>
<th>Girdle X Location X Induction Treatment</th>
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</thead>
<tbody>
<tr>
<td>total phenolics</td>
<td>0.98</td>
<td>3.64*</td>
<td>4.75**</td>
<td>16.40***</td>
<td>6.60****</td>
<td>0.58</td>
<td>2.73*</td>
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<td>catechin pentoside</td>
<td>1.01</td>
<td>5.29*</td>
<td>0.75</td>
<td>5.43**</td>
<td>3.26*</td>
<td>0.17</td>
<td>1.79</td>
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<tr>
<td>procyanidin trimer</td>
<td>1.68</td>
<td>0.93</td>
<td>0.61</td>
<td>5.68**</td>
<td>1.46</td>
<td>0.35</td>
<td>1.34</td>
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<tr>
<td>coumaroylquinic acid</td>
<td>2.62</td>
<td>1.14</td>
<td>3.83*</td>
<td>2.36</td>
<td>1.57</td>
<td>0.12</td>
<td>1.60</td>
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<tr>
<td>betuloside pentoside B</td>
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<td>1.77</td>
<td>1.81</td>
<td>3.82*</td>
<td>2.50*</td>
<td>1.13</td>
<td>2.11*</td>
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<tr>
<td>unknown</td>
<td>1.12</td>
<td>0.47</td>
<td>4.29**</td>
<td>1.74</td>
<td>3.97**</td>
<td>0.98</td>
<td>2.43*</td>
</tr>
<tr>
<td>platyphyloside</td>
<td>0.59</td>
<td>9.93**</td>
<td>0.99</td>
<td>1.23</td>
<td>4.65**</td>
<td>0.90</td>
<td>2.55*</td>
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<tr>
<td>diarylheptanoid hexoside</td>
<td>0.01</td>
<td>0.35</td>
<td>0.82</td>
<td>2.26</td>
<td>3.41**</td>
<td>0.83</td>
<td>1.51</td>
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<tr>
<td>centrolobol glycoside 1</td>
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<td>0.00</td>
<td>0.26</td>
<td>3.70*</td>
<td>1.93</td>
<td>2.87</td>
<td>1.47</td>
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<td>1.37</td>
<td>1.38</td>
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<td>0.89</td>
<td>3.26*</td>
<td>2.90*</td>
<td>0.30</td>
<td>0.63</td>
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</table>

*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
there were no differences in concentrations of procyandin trimer above versus below the girdle ($P=0.618$).

An interaction between girdling treatment and induction treatment affected concentrations of total phenolics and six individual phenolics (catechin pentoside, betuloside pentoside B, an unknown phenolic, platyphylloside, diarylheptanoid hexoside, centrolobol hexoside) (Table 4.1). In ungirdled trees, concentrations of total phenolics induced by mechanical wounding ($P=0.005$) and bronze birch borer ($P=0.050$) were higher than constitutive concentrations. However, in 1-year and 2-year girdled trees, constitutive concentrations of total phenolics were higher than concentrations induced by mechanical wounding ($P=0.022; 0.009$, respectively) or by bronze birch borer ($P=0.001; 0.001$, respectively). In ungirdled trees, concentrations of catechin pentoside, diarylheptanoid hexoside, and centrolobol hexoside induced by mechanical wounding ($P=0.009; 0.004; 0.050$, respectively) and bronze birch borer ($P=0.041; 0.045; 0.050$, respectively) were higher than constitutive treatments. In 1-year and 2-year girdled trees, constitutive concentrations of betuloside pentoside B, platyphylloside, diarylheptanoid hexoside, and centrolobol hexoside were higher than concentrations induced by mechanical wounding or by bronze birch borer inoculation.

A three-way interaction among girdling treatment, location, and induction treatment affected concentrations of total phenolics and three individual phenolics (betuloside pentoside B, an unknown, and platyphylloside) (Table 4.1). In general, mechanical wounding and bronze birch borer homogenate decreased concentrations of total phenolics, the unknown phenolic, and platyphylloside in 2-year girdled trees below the girdle (Fig. 4.2). Mechanical wounding and bronze birch borer homogenate
Figure 4.2. Concentrations of constitutive and induced total phenolics A above and B below a girdle in phloem of paper birch (Betula papyrifera). Probabilities for differences of the least squared means (PDIF) from analysis of variance (ANOVA) were used for statistical comparisons. Bars with different letters are significantly different ($\alpha = 0.05$).
decreased concentrations of betuloside pentoside B above the girdle in 1-year girdled trees and below the girdle in 2-year girdled trees.

**Multivariate analysis of phenolic chemistry**

1-year girdled, 2-year girdled, and ungirdled paper birch could not be differentiated from one another by principal component analysis of phenolic chemistry (Fig. 4.3A). The first two principal components explained 46.1% of the total variation. Location along the first PC axis was determined by catechin pentoside (eigen vector (EV): 0.30), platyphylloside (EV: 0.30), and diarylheptanoid hexoside (EV: 0.30). Location along the second PC axis was determined by betuloside (EV: 0.42), an unknown phenolic (EV: -0.33), and procyanidin trimer (EV: 0.31).

Phloem tissues above and below the girdle in 1-year and 2-year girded paper birch could not be distinguished from one another by multivariate analysis of phenolics (Fig. 4.3B). The first two principal components explained 49.2% of the total variation. Location along the first PC axis was determined by catechin pentoside (EV: 0.28), platyphylloside (EV: 0.28), and diarylheptanoid hexoside (EV: 0.28). Location along the second PC axis was determined by coumaroylquinic acid (EV: 0.36) and betuloside pentoside B (EV: -0.43).
Figure 4.3. Variation in phenolics A among girdling treatments of paper birch (*Betula papyrifera*) and B between phloem above and below the girdle in 1-year and 2-year girdled paper birch. Percentage of total variability explained by first two axes is shown.
Below the girdle of 1-year and 2-year girdled trees, bronze birch borer colonization was inversely related to induced concentrations of total phenolics and six individual phenolics (Fig. 4.4), and positively related to constitutive concentrations of total phenolics and five individual phenolics (Table 4.2). There were no relationships between bronze birch borer colonization and constitutive or induced levels of phenolics above the girdle.

Discussion

Trunk girdling decreased phloem resistance to bronze birch borer below the girdle, confirming that stress compromises resistance and supporting the hypothesis that paper birch resistance to bronze birch borer relies on current photosynthate that is translocated from the leaves to the trunk. More bronze birch borer colonized stem below than above the girdle in 2-year girdled trees, but there were no differences in levels of colonization in 1-year girdled trees or ungirdled trees below and above the girdle (Fig. 4.1). Previous research also found that girdled paper birch is more susceptible to bronze birch borer below the girdle than above (Anderson 1944) and that bronze birch borer larvae exhibit increased performance below the girdle than above (Herms 1991). In addition, girdled trees were more susceptible to bronze birch borer than ungirdled trees. Birch trees that were girdled for 2 years had the highest incidence of bronze birch borer colonization, followed by 1-year girdled trees, and then ungirdled trees.
Figure 4.4. Relationship between bronze birch borer (*Agrilus anxius*) colonization and induced phenolics below and above a 1-year or 2-year girdle in paper birch (*Betula papyrifera*). The difference in phenolic concentrations between an induction treatment and the unwounded (constitutive) control represent induced phenolics. Negative values indicate higher concentrations of phenolics in the control treatment and positive values indicate higher concentrations in the induction treatment.
Table 4.2. Correlations between bronze birch borer (*Agrilus anxius*) colonization and concentrations of constitutive and induced phenolics detected by HPLC-PDA in phloem of paper birch (*Betula papyrifera*) girdled for one or two years.

<table>
<thead>
<tr>
<th></th>
<th>Above girdle</th>
<th></th>
<th></th>
<th>Below girdle</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Constitutive</td>
<td>Induced</td>
<td>+ Induced</td>
<td>Constitutive</td>
<td>Induced</td>
<td>+ Induced</td>
</tr>
<tr>
<td>total phenolics</td>
<td>r(^a) 0.02</td>
<td>-11</td>
<td>-12</td>
<td>0.45</td>
<td>-0.55</td>
<td>-0.14</td>
</tr>
<tr>
<td></td>
<td>P(^b) 0.92</td>
<td>0.56</td>
<td>0.55</td>
<td>0.01</td>
<td>0.00</td>
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<td>catechin pentoside</td>
<td>r -0.02</td>
<td>-0.08</td>
<td>-0.12</td>
<td>0.30</td>
<td>-0.58</td>
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</tr>
<tr>
<td></td>
<td>P 0.89</td>
<td>0.65</td>
<td>0.52</td>
<td>0.11</td>
<td>0.00</td>
<td>0.16</td>
</tr>
<tr>
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<td>-0.01</td>
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<td>0.16</td>
<td>-0.32</td>
<td>-0.18</td>
</tr>
<tr>
<td></td>
<td>P 0.36</td>
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<td>0.37</td>
<td>0.4</td>
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<td>-0.08</td>
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<tr>
<td></td>
<td>P 0.89</td>
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<td>0.67</td>
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<td>betuloside pentoside B</td>
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<td>-0.13</td>
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<td></td>
<td>P 0.47</td>
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<td>0.51</td>
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<td></td>
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<td>0.72</td>
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<td>-0.21</td>
<td>0.26</td>
<td>-0.38</td>
<td>-0.21</td>
</tr>
<tr>
<td></td>
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<td>0.17</td>
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<td>0.28</td>
</tr>
<tr>
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<td>0.15</td>
<td>0.42</td>
<td>-0.38</td>
<td>0.01</td>
</tr>
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<td>P 0.8</td>
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<td>0.44</td>
<td>0.02</td>
<td>0.04</td>
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<td>0.55</td>
<td>-0.44</td>
<td>0.12</td>
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<td>0.20</td>
<td>0.27</td>
<td>0.00</td>
<td>0.01</td>
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</tr>
</tbody>
</table>

\(^a\)Pearson’s correlation coefficient.  
\(^b\)P-value of corresponding correlation above.  
\(^c\)Bold used to indicate significant correlations at \(\alpha=0.05\).
Girdling affected putative resistance mechanisms of paper birch. Girdling typically causes a shortage of photosynthate in plants parts below the girdle and accumulation above (Weaver and McCune 1959, Goldschmidt et al. 1985, Mataa et al. 1998, Li et al. 2003), which is consistent with our observations of reduced concentrations of total phenolics and rate of wound periderm growth in 1-year girdled paper birch below compared to above the girdle. However, girdling did not affect concentrations of total phenolics or rate of wound periderm growth of 2-year girdled trees above versus below the girdle, which may indicate severe carbon stress, possibly due to reduced capacity to maintain leaf area and photosynthesis (Schaffer et al. 1986, Roper and Williams 1989, Cheng et al. 2008).

Girdling affected induced birch responses over time. In 2-year girdled trees, concentrations of induced total phenolics were decreased below the girdle, but this was not observed in 1-year girdled trees or ungirdled trees. This indicates that carbon becomes more limited below the girdle over time. Typically, when local reserves are depleted, induced defenses rely on long-distance transport of photosynthate (Christiansen and Ericsson 1986, Berryman 1988, Bonello et al. 2006); however, in girdled trees, long-distance transport is cut off below the girdle and the ability to maintain induced defenses could become constrained (Bonello et al. 2006). Additionally, a reduction in induced concentrations of simple phenolics in paper birch may indicate that they serve as precursors to more complex molecules (Boerjan et al. 2003, Dixon et al. 2005). Upon wounding of plant tissue, phenolics are released from vacuoles and can polymerize to form lignin, which is an important component of wound periderm and wound healing (Beckman et al. 1974, Beckman 2000).
Levels of bronze birch borer colonization were inversely related to levels of induced phenolics below the girdle (Fig. 4.4). Similar to the relationship between bark beetle and pine defenses, one explanation of this pattern could be that when high densities of bronze birch borer larvae breach a critical threshold, they overwhelm the tree’s ability to defend itself (Raffa and Berryman 1983, Raffa et al. 2008). Below this critical threshold (i.e. low density) beetles do not affect production and allocation of defenses, and the tree maintains its defended status. However, an alternative explanation could be that as carbon stress of the birch increases, induction of carbon-based defenses may become limited, resulting in increased susceptibility and increased levels of beetle colonization. Under this latter scenario, non-stressed trees are able to maintain higher levels of induced defenses resulting in low levels of insect colonization. In contrast, levels of bronze birch borer colonization and induced phenolics were unrelated above the girdle (Fig. 4.4). Changes in concentrations of other chemicals in phloem, such as nutrients (Haack and Slansky 1987), defensive proteins (Poyla 2003), tannins (Moilanen and Salminen 2008), or lignin (Wainhouse et al. 1990, Hagerman and Butler 1991) may mediate the effectiveness of induced defenses and uncouple the inverse relationship between induced defenses and larval colonization that was observed below the girdle.

Girdled and ungirdled trees, as well as phloem above and below the girdle, could not be differentiated from one another by multivariate analysis of phenolic chemistry (Fig. 4.3AB). In contrast, phenolic profiles between species of birch are distinctly different (Chapters 2 and 3). This indicates that interspecific variation in phenolic composition is greater than intraspecific variation in phenolic composition imposed by physiological stress.
The trees in this study, including the ungirdled control trees, appear to have been stressed prior to any treatments we applied. All trees had very slow rates of wound periderm formation and low levels of phenolics. On average, there was no growth of wound periderm in ungirdled control trees after 10 months whereas in a comparable study wound periderm growth was 2.5 mm after two months (Chapter 3). Additionally, concentration of total phenolics in ungirdled control trees was 45 mg/g compared to 129 mg/g in a comparable study (Chapter 3). The stressor underlying this lack of plant vigor could be drought as the Palmer drought severity index indicates a net moderate drought in North Central Wisconsin from 2004 to 2009 (http://www.drought.noaa.gov). Drought stress reduces source strength (i.e. pool of available photosynthate) by decreasing stomatal conductance and photosynthesis (Flexas and Medrano 2002). Therefore, drought stress likely dampened the effects of girdling on source-sink interactions, specifically, the \textit{a priori} expectation for accumulation of photosynthate above the girdle.

Historically, stress has been repeatedly associated with increased susceptibility of paper birch to colonization by bronze birch borer (Balch and Prebble 1940, Anderson 1944, Barter 1957, Jones et al. 1993). Our findings confirm that stress compromises resistance of paper birch to bronze birch borer colonization and supports the hypothesis that the flux of current photosynthate from leaves to trunk provides the energy to drive defensive responses. Our findings also indicate that in interactions between coevolved trees and wood-boring insects, maintenance of tree vigor is important in bottom-up regulation of populations of wood-boring insects.
Acknowledgements

I thank William Mattson and the US Forest Service in Rhinelander, WI for access to birch. I thank Bruce Birr, Priyadarshani Loess, Daniel Herms, Bryant Chambers, and Samuel Discua for assistance with fieldwork and Priyadarshani Loess and Loren Rivera for assistance with sample preparation. I thank Pierluigi Bonello and Larry Phelan for use of their HPLCs and Robin Taylor and Bert Bishop for statistical consultation.
References


Minitab Inc. 2007. Minitab 15 statistical software, State College, PA.


Chapter 5

Patterns of bronze birch borer colonization and paper birch phloem chemistry under elevated CO$_2$ and O$_3$

Abstract

Elevated levels of CO$_2$ and O$_3$ can affect plant chemistry, which can alter plant resistance to herbivores. We studied their effects on the interaction between paper birch (Betula papyrifera) and bronze birch borer (Agrilus anxius), a wood-boring beetle endemic to North America, at the Aspen Free Air CO$_2$ Enrichment (FACE) facility in northern WI, USA. Throughout the four-year study, bronze birch borer colonization was highest in trees exposed to elevated O$_3$ in combination with ambient CO$_2$. However, elevated CO$_2$ ameliorated the negative effects of elevated O$_3$, as bronze birch borer colonization was much lower in trees exposed to this treatment combination. Over time, levels of bronze birch borer colonization increased in trees exposed to elevated CO$_2$ in combination with ambient O$_3$, but there was high variation in levels of colonization among rings. Elevated CO$_2$ and O$_3$ altered phloem chemistry of paper birch, but phytochemical variation did not explain patterns of bronze birch borer colonization. Elevated CO$_2$ decreased concentration of nitrogen in phloem but did not affect concentrations of amino acids, phenolics, tannins, or lignin. Elevated O$_3$ and the interaction between CO$_2$ and O$_3$ altered
concentrations of three phenolics, but most phytochemicals were not affected. Experimental wounding and bronze birch borer homogenate elicited increased lignin concentration in phloem as well as changes in concentrations of individual phenolics and amino acids. The results suggest that on-going changes in atmospheric composition could compromise resistance of paper birch to bronze birch borer, possibly facilitating outbreaks, which could result in altered distribution of paper birch of North American boreal forests.
Introduction

Concentrations of atmospheric carbon dioxide ($CO_2$) have recently reached 386.8 ppm (ESRL/NOAA 2010), which is 40% higher than concentrations at the beginning of the industrial revolution (Pearson and Palmer 2000). Concentrations of tropospheric ozone ($O_3$) have also increased and are on average 38% higher than pre-industrial levels (Denman et al. 2007). Since plants fix $CO_2$ from the atmosphere to synthesize biomolecules, compositional changes in the atmosphere could drastically affect plant biochemistry and physiology, affecting ecological interactions at population (Coviella and Trumble 1999, Percy et al. 2002, Stiling et al. 2009), community (Morgan et al. 2007, Hillstrom and Lindroth 2008), and ecosystem levels (Saxe et al. 1998, Lindroth 2010), including plant-herbivore interactions.

There has been extensive research on the effects of elevated $CO_2$ on plant-insect interactions, predominantly involving folivores (Peñuelas and Estiarte 1998). Herbivore response to plants growing in enriched $CO_2$ atmospheres has been variable as increases, decreases, and no change in herbivore preference and performance have been observed (Bezemer and Jones 1998, Lindroth 2010). Plants typically respond to elevated $CO_2$ by increasing C:N content (Drake et al. 1997, Beedlow et al. 2004), increasing photosynthesis and growth (“fertilizer effect”) (Beedlow et al. 2004), and increasing water-use efficiency (Lawlor and Mitchell 1991, Drake et al. 1997). Changes in pools of secondary metabolites have been variable as concentrations of tannins and low molecular weight phenolics generally increase but concentrations of terpenes are variable (Peñuelas and Estiarte 1998). The growth differentiation balance hypothesis (GDBH) predicts that
environmental resource variability, including variability in concentrations of CO$_2$, creates tradeoffs in partitioning of carbon to secondary metabolites, growth, and storage because of the dynamic relationship between source supply (i.e. supply of photosynthate) and sink strength (i.e. growth and storage demand) (Herms and Mattson 1992). Specifically, GDBH predicts increased partitioning to phenolics that is proportional to the surplus between sink demands and supply of photosynthate when photosynthesis and growth increase in response to elevated CO$_2$ or when photosynthesis increases but growth is unchanged (Mattson et al. 2005). However, if photosynthesis is not affected by elevated CO$_2$ but growth increases, decreased partitioning to phenolics will occur, and if photosynthesis and growth are not affected, then there will be no change in partitioning to phenolics (Mattson et al. 2005).

Generally, the positive impact of elevated O$_3$ on herbivore performance is mitigated by simultaneous exposure to elevated CO$_2$ (Valkama et al. 2007) because CO$_2$ ameliorates the detrimental effects of O$_3$ on plants (Rao et al. 1995, Valkama et al. 2007). Elevated CO$_2$ decreases stomatal conductance and thus oxidative stress induced by O$_3$, and reduces carbon stress associated with elevated O$_3$ (Allen 1990, Lindroth 2010).

Although numerous experiments have investigated the independent and/or interactive effects of elevated CO$_2$ and O$_3$ on insect-plant interactions, to our knowledge none of these have included interactions between wood-boring insects and their hosts. Historically in North America, widespread tree mortality by periodic outbreaks of wood-boring insects has been associated with tree stress (Balch and Prebble 1940, Anderson 1944, Barter 1957, Cote and Allen 1980, Jones et al. 1993, Stephen et al. 2001). This indicates that changes in host plant resistance can markedly affect population dynamics of wood-boring insects and that ongoing climate change, via changes in host plant resistance, might provoke outbreaks. Elevated ozone, specifically, has been implicated to predispose woody angiosperms to colonization by wood-boring beetles, leading to decline and mortality of trees (Manion 1981).

We investigated the effects of elevated CO$_2$ and O$_3$ on the interaction between paper birch (*Betula papyrifera* Marshall) and bronze birch borer (*Agrilus anxius* Gory) (Coleoptera: Buprestidae), which are both endemic to North America. This is an instructive model system because paper birch is a widely distributed boreal species that is hypothesized to be sensitive to climate stress (Jones et al. 1993, Haack 1996). Historically, environmental (Balch and Prebble 1940, Jones et al. 1993), biotic (Balch and Prebble 1940), and physical (Anderson 1944, Barter 1957) stress have been
implicated in compromising paper birch resistance to bronze birch borer, leading to birch mortality. However, the mechanism(s) underlying the shift to increased susceptibility of paper birch to bronze birch borer are not known, although both physical and chemical plant traits may be involved. Therefore, in this study, we examined the effects of elevated CO$_2$ and O$_3$ on patterns of bronze birch borer colonization as well as potential mechanisms of resistance, including constitutive and induced phytochemistry (free amino acids, phenolics, tannins, lignin, nitrogen, and carbon) and growth rate of wound periderm (i.e. callus tissue). We predicted that fewer bronze birch borer would colonize paper birch exposed to elevated CO$_2$ because of increased resistance of paper birch (i.e. increased concentrations of phenolics and decreased concentrations of nitrogen) and that more bronze birch borer would colonize paper birch exposed to elevated O$_3$ (a plant stressor) due to decreased birch resistance. Finally, we predicted that elevated CO$_2$ would ameliorate the negative effects of elevated O$_3$ on paper birch resistance, resulting in decreased levels of bronze birch borer colonization when compared to levels of colonization in trees exposed to elevated O$_3$ alone.

Methods

Aspen FACE site

This experiment was conducted at the Free Air CO$_2$ Enrichment (Aspen FACE) facility in north-central Wisconsin, U.S.A. (89.7° W, 45.7° N), which contains 12 30-m diameter rings composed of aspen, birch, and maple trees planted on a 1 X 1 m spacing.
One half of each ring is planted with trembling aspen (*Populus tremuloides*) and the other half is further subdivided into quarters of which one quarter contains aspen and maple (*Acer saccharum*) and the other contains aspen and paper birch. Trees were planted in July of 1997 and have been exposed to elevated concentrations of CO$_2$ and/or O$_3$ for their entire life history (Dickson et al. 2000). The rings are arranged in a 2 X 2 factorial randomized block design with two levels of CO$_2$ (ambient and 560 ppm) and two levels of O$_3$ (ambient and 1.5X ambient). Elevated CO$_2$ concentrations are those predicted to occur by 2060, and elevated O$_3$ concentrations model current seasonal and diurnal fluctuations in urban regions of the western Great Lakes region (Pinkerton and Lefohn 1987, Karnosky et al. 1996).

*Bronze birch borer colonization*

From 2005-2008, levels of bronze birch borer colonization were estimated on all paper birch at the FACE experiment by quantifying larval galleries and adult emergence (N=877). Serpentine larval galleries, which are externality visible through the thin bark of paper birch, and D-shaped exit holes, which are made by emerging adults, were counted. Old exit holes were circled using a wax pencil prior to May adult emergence to separate emergence holes from current generation adults from previous generation adults.

Ninety-six paper birch, which had phloem treatments applied to them previously (explained below), were felled on 11 May 2009. A 1.5 m log located 1 m above ground level on the stem of the tree was dissected to expose larval galleries and internally
examine larval galleries. All larval galleries were counted and measured using a string to trace gallery length to the nearest mm.

**Constitutive and induced phloem treatments**

Phloem samples were excised in 2007 and 2008 to analyze constitutive chemistry and induced responses of paper birch, respectively. In order to analyze constitutive phenolic chemistry of paper birch phloem, one phloem plug was excised using a 10 mm arch punch approximately 1.3 m above ground level from eight randomly chosen trees in each ring (N = 96). Trees were sampled on 9 July 2007 to correspond to the natural phenology of bronze birch borer neonate feeding (Akers and Nielsen 1990).

In order to analyze induced phytochemical responses, induction treatments were applied on 22 June 2008 to the same trees as in 2007. Induction treatments were applied to three sites on the stem, approximately 1.3 m above ground level. Treatments were arranged to correspond with the points of an equilateral triangle, were 10 cm apart, and applied randomly within each arrangement. Two sites were wounded using a sterilized 5 mm punch that removed a phloem plug. In order to analyze localized induced responses specific to bronze birch borer, 25 mg freeze-dried bronze birch borer larval homogenate was inserted into one wound. The wound without bronze birch homogenate was used to analyze localized induced responses to mechanical damage. Both wounds were covered with a 2 % agar plug, parafilm and duct tape. The third site remained unwounded in order to quantify constitutive phloem chemistry and to serve as a baseline in order to determine induced responses. Phloem samples were excised from all three treatment locations 72 h
after wounding using a 15 mm punch, which was centered around smaller elicitation wounds in order to sample surrounding tissue. Samples were flash frozen in liquid nitrogen and stored at -80°C until they were ground for chemical analysis.

Approximately two months after excising phloem samples in 2008, the diameter of each wound was measured using calipers. Growth rate of wound periderm (mm per day) was calculated by subtracting the final diameter of the wound from the original diameter and dividing by the number of days that wound periderm could grow.

*Analysis of low molecular weight phenolics*

Identification and quantification of low molecular weight phenolics were completed as in chapter 2. Constitutive phenolics in tissue of phloem samples collected in 2007 were extracted from 100 mg of ground phloem in two aliquots of 500 µL of HPLC grade methanol (Fisher, Pittsburgh, PA) for 24 h at 4°C (Bonello and Blodgett 2003). The extracts were combined and stored at -20°C until analysis by high-performance liquid chromatography (HPLC). Induced phenolics in tissue of phloem samples collected in 2008 were extracted from 50 mg of ground phloem in two aliquots of 250 µL of HPLC grade methanol.

Identification of phenolics was completed using a Varian 212-LC chromatography pump equipped with a ProStar 410 HPLC autosampler, a Varian Polaris™ RP18, 4.6 X 150 mm column, and a Varian 500-MS ion trap mass spectrometer operating in the ESI mode. Scans were completed from 100-1400 m/z to measure [M-H] ions. Quantification of phenolics was completed using a Waters 2690 separations module.
(Milford, MA), equipped with a Waters Xterra™ RP18, 5 µm, a 4.6 X 150 mm column, a Waters Xterra™ RP18, a 3.9 µm, 3.0 X 20 mm guard column, and a 996 photodiode array detector. The binary mobile phase consisted of 2% glacial acetic acid in HPLC-grade water and 2% glacial acetic acid in HPLC-grade methanol. The solvent program was as follows (percentages represent proportion of methanol): 0% (0-2 min); 0-10% (2-4 min); 10-48% (4-20 min); 48-100% (20-38 min); 100% (38-39 min); 100-0% (39-41 min). The flow rate was 1 mL/min, and the injection volume for each sample was 10 µL.

Analyzed phenolics were quantified against the following commercial standards: catechin (Alexis Biochemicals, San Diego, CA, USA), chlorogenic acid (MP Biomedicals, Solon, OH, USA), and salicin (Sigma Aldrich, Milwaukee, WI, USA). The glycosides catechin pentoside, betuloside, betuloside pentoside, and diarylheptanoids were expressed as salicin equivalents. Catechin and procyanidins were expressed as catechin equivalents. Coumaroylquinic acid, platyphylloside and unknown peaks 9-12 were expressed as chlorogenic acid equivalents. Compounds were expressed as mg per g fresh weight.

Total phenolics, condensed tannins, and lignin

Tissue from phloem samples collected in 2008 was also analyzed to quantify constitutive and induced concentrations of condensed tannins, Folin-Denis total phenolics, and lignin. Condensed tannins and total phenolics were analyzed as in Nitao et al. 2001 using colorimetric analysis on a Rapid Flow Analyzer (Astoria-Pacific, OR). Briefly, ground phloem samples were extracted in 50% methanol. Total phenolics were
analyzed using the Folin-Denis assay in which samples were diluted with 1.5% sodium lauryl sulfate and mixed with a sodium carbonate solution and Folin-Denis reagent within the Rapid Flow Analyzer. Total phenolics were analyzed at 580 nm using tannic acid as a standard for quantification. To analyze condensed tannins, 5% concentrated hydrochloric acid in butanol and 2% ammonium sulfate in 2N hydrochloric acid were added to sample extracts. Condensed tannins were analyzed at 750 nm using quebracho tannin as a standard for quantification. Concentrations of total phenolics and tannins were expressed as mg per g fresh weight.

Lignin concentration was analyzed as described by Bonello and Blodgett 2003. A series of solvents, acids, and bases removed soluble phenolics and phenolics bound in the cell wall. The remaining lignin was quantified at 280 nm in a spectrophotometer using lignin as an external standard (Aldrich Chemical Co., Milwaukee, WI, USA). Concentrations of lignin were expressed as mg per g fresh weight.

C:N and amino acids

Constitutive concentrations of carbon and nitrogen from tissue from phloem samples collected in 2008 were also analyzed. Five mg of ground phloem tissue were used to quantify the C:N. Analysis was completed using a Thermo Electron Corporation (CE Instruments) Flash EA-1112 C-N Analyzer with an AS 3000 Autosampler.

Constitutive and induced concentrations of free amino acids in tissue from phloem samples collected in 2008 were analyzed. Twenty-five mg of phloem tissue was extracted in 500 uL 0.01 N hydrochloric acid. Free amino acids in the extract were
derivatized using the EZ:faast® GC-MS physiological amino acid analysis kit (Phenomenex Inc., Torrence, CA). GC-MS was completed using an Agilent 6890 Series GC System equipped with a 5973 Mass Selective Detector, a 7683B Series Injector, and a 7683 Series Autosampler. All free amino acids except GABA were identified and quantified using internal and external amino acid standards included in the kit. GABA was identified and quantified using GABA (Sigma, Saint Louis, MO, USA) as an external standard. Concentrations of amino acids were expressed as nmol per mg fresh weight.

Statistical models and analyses

The experimental design for analysis of phenolics from 2007, carbon and nitrogen from 2008, and bronze birch borer colonization was a randomized complete block design. A 2 X 2 factorial arrangement of CO₂ and O₃ (ambient and elevated levels) were arranged in three blocks. Blocks were random effects.

The experimental design for analysis of phenolics, tannin, lignin, and amino acids from 2008 was a split plot design. As before, there was a 2 X 2 factorial arrangement of CO₂ and O₃ at the whole plot level replicated in three blocks. Phloem treatment was the subplot factor. Trees were nested within block*CO₂*O₃. The statistical model for the split plot design was

\[ Y_{ijklm} = \mu + B_i + CO2_j + O3_k + CO2O3_{jk} + \gamma_{ijk} + P_l + CO2P_{jl} + O3P_{kl} + CO2O3P_{jkl} + T_m(BCO2O3_{ijk}) + \epsilon_{ijklm} \]
where $Y_{ijkl}$ was the response of block $i$, CO$_2$ treatment $j$, O$_3$ treatment $k$, and phloem treatment $l$. Block ($B_i$) and tree ($T_m$) were random effects. All other terms were considered fixed which included CO$_2$ treatment ($CO2_j$), O$_3$ treatment ($O3_k$), phloem treatment ($P_l$) and their interactions. $\gamma_{ijk}$ was the error for the whole plot unit, and the residual ($\epsilon_{ijkl}$) was the error term for the split plot unit.

Data were analyzed by analysis of variance (ANOVA; PROC MIXED) using SAS 9.1 (SAS Institute. 1999). The low number of replicates of the FACE experimental design increases the probability for type II errors (Filion et al. 2000). Therefore, $P$-values $\leq 0.10$ were considered significant at the whole plot level (Kopper and Lindroth 2003). Following a significant $F$ test, probabilities for differences of the least squared means (PDIFF) were used for comparisons of phloem treatments, CO$_2$ by O$_3$ interactions, CO$_2$ by phloem treatment interactions, O$_3$ by phloem treatment interactions, and CO$_2$ by O$_3$ by phloem treatment interactions.

We used two approaches to analyze relationships between bronze birch borer colonization and plant traits (e.g. growth rate of wound periderm, concentration of phytochemical, tree diameter, etc.). ANOVA (PROC GLM) was used to test differences among trees characterized by different levels of bronze birch borer colonization. Each tree was placed into one of four groups: low, medium, high, or no bronze birch borer colonization. Trees with low colonization had one larval gallery. Trees with medium colonization had two larval galleries and/or one or two exit holes. Trees with high colonization had three or more galleries and/or three or more exit holes. Trees with no bronze birch borer colonization had no larval galleries and no exit holes. The data were analyzed as a completely randomized design and each tree was an experimental unit.
Additionally, Pearson’s correlation coefficients were used to quantify the relationship between total gallery length per tree with concentrations of phytochemicals and rate of wound periderm growth. Specifically, constitutive, induced, and total concentrations of phytochemicals were correlated with total gallery length. The difference in phenolic concentrations between the induction treatment inoculated with bronze birch borer and the unwounded (constitutive) control represented induced phenolics. Total concentrations were those present in induction treatments inoculated with bronze birch borer and mathematically are determined by the summation of constitutive and induced concentrations.

Results

Patterns of bronze birch borer colonization

In 2005, 2006, and 2007, an interaction between CO₂ and O₃ affected patterns of bronze birch borer colonization (Fig. 5.1). Bronze birch borer colonized more trees fumigated with elevated O₃ and ambient CO₂ than all other treatment combinations during these years. By 2008, levels of bronze birch borer colonization were also high in trees fumigated with elevated CO₂ and ambient O₃. However, there was high variation in levels of colonization among rings exposed to this treatment combination. CO₂ and O₃ did not affect total gallery length of bronze birch borer larvae per tree.
Figure 5.1. Patterns of bronze birch borer (*Agrilus anxius*) colonization of paper birch (*Betula papyrifera*) grown in ambient (Lo) or elevated (Hi) levels of CO$_2$ and O$_3$ at the Aspen FACE site in Rhinelander, WI, USA from 2005-2008. Error bars represent pooled standard error from analysis of variance.
Elevated CO\textsubscript{2} and O\textsubscript{3} altered constitutive and induced defenses of birch phloem (Table 5.1). In 2007, elevated O\textsubscript{3} decreased constitutive concentrations of betuloside \((P=0.089)\). CO\textsubscript{2} and O\textsubscript{3} did not affect constitutive concentrations of other individual or total phenolics in 2007. In 2008, elevated CO\textsubscript{2} reduced the rate of wound periderm growth, and elevated O\textsubscript{3} increased levels of betuloside (Table 5.1). Additionally in 2008, an interaction between CO\textsubscript{2} and O\textsubscript{3} affected concentrations of betuloside pentoside A, betuloside B, and three diarylheptanoids: centrolobol glycoside 1, centrolobol glycoside 2, and centrolobol hexoside. Elevated CO\textsubscript{2} in combination with ambient O\textsubscript{3}, and ambient CO\textsubscript{2} in combination with elevated O\textsubscript{3}, decreased concentrations of betuloside pentoside A and B but increased concentrations of the three diarylheptanoids. Additionally, elevated O\textsubscript{3} decreased concentrations of procyanidin trimer and catechin induced by bronze birch borer homogenate. CO\textsubscript{2}, O\textsubscript{3} and their interactions with induction treatments did not affect concentrations of Folin Denis total phenolics, tannins, lignin, or growth rate of wound periderm.

Mechanical wounding and bronze birch borer homogenate altered levels of individual phenolics and lignin (Table 5.1). Specifically, mechanical wounding and bronze birch borer homogenate increased concentrations of platyphylloside, diarylheptanoid hexoside, centrolobol glycoside 1, centrolobol glycoside 2, and centrolobol hexoside, but decreased concentrations of procyanidin dimer and an unknown compound. Bronze birch borer homogenate also increased concentrations of lignin.
Table 5.1. *F*-values from analysis of variance (ANOVA) of putative defenses in phloem of paper birch (*Betula papyrifera*) to carbon dioxide (CO$_2$), ozone (O$_3$), and induction treatments (ITrt) at the Aspen FACE site in Rhinelander, WI, USA during 2008.

<table>
<thead>
<tr>
<th>putative defense</th>
<th>CO$_2$</th>
<th>O$_3$</th>
<th>CO$_2$ X O$_3$</th>
<th>ITrt</th>
<th>CO$_2$ X ITrt</th>
<th>O$_3$ X ITrt</th>
<th>CO$_2$ X O$_3$ X ITrt</th>
</tr>
</thead>
<tbody>
<tr>
<td>procyanidin trimer</td>
<td>0.28</td>
<td>0.21</td>
<td>1.08</td>
<td>1.71</td>
<td>0.77</td>
<td>4.19***</td>
<td>0.86</td>
</tr>
<tr>
<td>catechin</td>
<td>0.66</td>
<td>0.00</td>
<td>0.66</td>
<td>0.16</td>
<td>2.19</td>
<td>2.95**</td>
<td>0.55</td>
</tr>
<tr>
<td>procyanidin dimer</td>
<td>0.25</td>
<td>0.19</td>
<td>2.11</td>
<td>4.91***</td>
<td>0.57</td>
<td>1.86</td>
<td>1.04</td>
</tr>
<tr>
<td>betuloside pentoside A</td>
<td>0.05</td>
<td>0.09</td>
<td>7.71**</td>
<td>1.57</td>
<td>3.72**</td>
<td>0.87</td>
<td>0.91</td>
</tr>
<tr>
<td>betuloside</td>
<td>0.40</td>
<td>3.60*</td>
<td>0.02</td>
<td>0.58</td>
<td>0.51</td>
<td>0.19</td>
<td>0.34</td>
</tr>
<tr>
<td>betuloside pentoside B</td>
<td>1.08</td>
<td>0.83</td>
<td>3.81*</td>
<td>0.57</td>
<td>0.53</td>
<td>0.21</td>
<td>0.37</td>
</tr>
<tr>
<td>unknown</td>
<td>0.00</td>
<td>0.18</td>
<td>0.45</td>
<td>2.34*</td>
<td>1.34</td>
<td>0.18</td>
<td>1.29</td>
</tr>
<tr>
<td>platyphyloside</td>
<td>1.58</td>
<td>2.28</td>
<td>0.12</td>
<td>10.64****</td>
<td>2.31</td>
<td>0.40</td>
<td>0.36</td>
</tr>
<tr>
<td>diarylheptanoid hexoside</td>
<td>0.48</td>
<td>0.33</td>
<td>0.13</td>
<td>5.44**</td>
<td>1.55</td>
<td>1.15</td>
<td>0.64</td>
</tr>
<tr>
<td>centrolebol glycoside 1</td>
<td>0.52</td>
<td>1.19</td>
<td>7.93**</td>
<td>5.15***</td>
<td>1.10</td>
<td>0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>centrolebol glycoside 2</td>
<td>0.47</td>
<td>1.47</td>
<td>6.10***</td>
<td>5.82***</td>
<td>1.33</td>
<td>1.17</td>
<td>0.06</td>
</tr>
<tr>
<td>centrolebol hexoside</td>
<td>0.04</td>
<td>0.71</td>
<td>5.33**</td>
<td>14.02****</td>
<td>0.19</td>
<td>0.56</td>
<td>1.12</td>
</tr>
<tr>
<td>total phenolics$^a$</td>
<td>0.08</td>
<td>0.86</td>
<td>1.43</td>
<td>0.90</td>
<td>0.45</td>
<td>0.38</td>
<td>1.12</td>
</tr>
<tr>
<td>tannins</td>
<td>0.08</td>
<td>0.17</td>
<td>1.18</td>
<td>1.47</td>
<td>0.55</td>
<td>1.10</td>
<td>0.97</td>
</tr>
<tr>
<td>lignin</td>
<td>0.01</td>
<td>1.35</td>
<td>0.23</td>
<td>11.67****</td>
<td>1.66</td>
<td>1.53</td>
<td>0.47</td>
</tr>
<tr>
<td>growth rate of wound periderm</td>
<td>4.65*</td>
<td>2.25</td>
<td>0.88</td>
<td>0.61</td>
<td>0.37</td>
<td>2.32</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*$P<0.1$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

$^a$As detected by Folin-Dennis assay.
Induction treatments did not affect concentrations of Folin-Dennis total phenolics, condensed tannins, or growth rate of wound periderm.

*C:N and amino acids*

Elevated CO$_2$ and O$_3$ altered constitutive concentrations of nitrogen and carbon in birch phloem. Elevated CO$_2$ reduced constitutive concentrations of nitrogen ($P=0.035$). Elevated CO$_2$ in combination with ambient O$_3$ increased the carbon to nitrogen ratio ($P=0.075$).

Elevated CO$_2$, O$_3$, and their interactions with induction treatments modified concentrations of free amino acids in birch phloem (*Table 5.2*). Elevated O$_3$ decreased concentrations of tyrosine. Elevated CO$_2$ increased concentrations of phenylalanine but decreased concentrations of total amino acids and aspartic acid induced by mechanical wounding and/or bronze birch borer homogenate. Elevated O$_3$ decreased concentrations of serine and glutamic acid induced by bronze birch borer. Concentrations of GABA, valine, and serine were affected by an interaction among CO$_2$, O$_3$, and induction treatment. Elevated CO$_2$ in combination with ambient O$_3$, and ambient CO$_2$ in combination with elevated O$_3$, increased induced concentrations of valine but decreased induced concentrations of GABA.

Mechanical wounding and/or bronze birch borer homogenate affected concentrations of almost all free amino acids (*Table 5.2*). Specifically, mechanical wounding and bronze birch borer homogenate decreased concentrations of nine amino acids (glycine, isoleucine, threonine, GABA, aspartic acid, lysine, histidine, tyrosine and
Table 5.2. F-values from analysis of variance (ANOVA) of concentrations of free amino acids in phloem of paper birch (*Betula papyrifera*) to carbon dioxide (CO$_2$), ozone (O$_3$), and induction treatments (ITrt) at the Aspen FACE site in Rhinelander, WI, USA during 2008.

<table>
<thead>
<tr>
<th>amino acid</th>
<th>CO$_2$</th>
<th>O$_3$</th>
<th>O$_3$ X ITrt</th>
<th>CO$_2$ X ITrt</th>
<th>O$_3$ X ITrt</th>
<th>CO$_2$ X O$_3$ X ITrt</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>1.12</td>
<td>0.02</td>
<td>0.24</td>
<td>43.01****</td>
<td>0.71</td>
<td>1.12</td>
</tr>
<tr>
<td>glycine</td>
<td>0.47</td>
<td>0.20</td>
<td>1.46</td>
<td>3.92**</td>
<td>2.04</td>
<td>0.99</td>
</tr>
<tr>
<td>valine</td>
<td>0.63</td>
<td>0.31</td>
<td>0.93</td>
<td>3.65**</td>
<td>1.82</td>
<td>0.47</td>
</tr>
<tr>
<td>leucine</td>
<td>0.78</td>
<td>0.66</td>
<td>1.04</td>
<td>3.80**</td>
<td>0.12</td>
<td>1.89</td>
</tr>
<tr>
<td>isoleucine</td>
<td>0.72</td>
<td>0.19</td>
<td>0.82</td>
<td>6.35***</td>
<td>0.71</td>
<td>0.25</td>
</tr>
<tr>
<td>threonine</td>
<td>0.53</td>
<td>0.09</td>
<td>2.34</td>
<td>4.55***</td>
<td>1.70</td>
<td>0.59</td>
</tr>
<tr>
<td>GABA</td>
<td>1.01</td>
<td>0.01</td>
<td>0.60</td>
<td>15.77****</td>
<td>2.26</td>
<td>0.11</td>
</tr>
<tr>
<td>serine</td>
<td>0.53</td>
<td>0.38</td>
<td>0.20</td>
<td>0.45</td>
<td>2.15</td>
<td>4.39**</td>
</tr>
<tr>
<td>proline</td>
<td>1.04</td>
<td>0.84</td>
<td>0.35</td>
<td>27.12****</td>
<td>1.45</td>
<td>2.03</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>1.54</td>
<td>0.14</td>
<td>0.09</td>
<td>6.61***</td>
<td>3.14**</td>
<td>0.91</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>0.05</td>
<td>0.66</td>
<td>0.04</td>
<td>2.34*</td>
<td>1.36</td>
<td>3.21**</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>0.74</td>
<td>3.35</td>
<td>1.63</td>
<td>9.64****</td>
<td>3.93**</td>
<td>0.84</td>
</tr>
<tr>
<td>lysine</td>
<td>0.22</td>
<td>1.25</td>
<td>3.13</td>
<td>27.27****</td>
<td>1.49</td>
<td>3.02</td>
</tr>
<tr>
<td>histidine</td>
<td>1.03</td>
<td>1.15</td>
<td>0.15</td>
<td>22.67****</td>
<td>0.55</td>
<td>0.97</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.26</td>
<td>4.31*</td>
<td>0.48</td>
<td>20.01****</td>
<td>0.04</td>
<td>1.87</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.07</td>
<td>0.66</td>
<td>0.27</td>
<td>13.77****</td>
<td>0.28</td>
<td>0.39</td>
</tr>
<tr>
<td>total AAs</td>
<td>1.04</td>
<td>0.00</td>
<td>0.08</td>
<td>1.14</td>
<td>3.54**</td>
<td>2.19</td>
</tr>
<tr>
<td>essential AAs</td>
<td>0.10</td>
<td>0.75</td>
<td>2.57</td>
<td>30.70****</td>
<td>2.11</td>
<td>0.78</td>
</tr>
</tbody>
</table>

*P<0.1; **P<0.05; ***P<0.01; ****P<0.001.

GABA, gamma-aminobutyric acid.
tryptophan) and essential amino acids, but increased concentrations of four amino acids (leucine, proline, glutamic acid, and phenylalanine). Additionally, bronze birch borer homogenate increased induced concentrations of alanine and valine.

*Relationship between bronze birch borer colonization and plant traits*

Levels of bronze birch borer colonization, as indicated by total gallery length per tree, were correlated with both defensive and nutritive traits (Table 5.3). Bronze birch borer colonization was inversely correlated to induced levels of procyanidin trimer and catechin. Bronze birch borer colonization was positively correlated to constitutive levels of two phenolics (unknown 1, unknown 3), induced levels of one phenolic (unknown 4), and total levels of three phenolics (unknown 3, unknown 4, platyphylloside). Bronze birch borer colonization was also inversely correlated to constitutive levels of valine, tryptophan, and essential amino acids as well as total levels of valine and proline. Levels of bronze birch borer colonization were not related to growth rate of wound periderm, tree diameter, or other phytochemicals.

Paper birch with medium or high levels of bronze birch borer colonization were characterized by high concentrations of constitutive lignin ($P=0.009$), high concentrations of carbon ($P=0.091$), and high induced concentrations of aspartic acid ($P=0.054$). Paper birch with medium levels of bronze birch borer had high constitutive and induced concentrations of catechin pentoside ($P=0.034$, 0.036, respectively). Paper birch with no colonization by bronze birch borer had high constitutive levels of phenylalanine ($P=0.069$).
Table 5.3. Correlations between bronze birch borer (*Agrilus anxius*) colonization and constitutive and induced phenolics in phloem tissues of paper birch (*Betula papyrifera*). Only significant correlations ($\alpha = 0.05$) are shown.

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Constitutive</th>
<th>Induced</th>
<th>Constitutive + Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>procyanidin trimer</td>
<td>$r^a$</td>
<td>-0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P^b$</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>catechin</td>
<td>$r$</td>
<td>-0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>unknown phenolic 1</td>
<td>$r$</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>unknown phenolic 3</td>
<td>$r$</td>
<td>0.39</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>unknown phenolic 4</td>
<td>$r$</td>
<td>0.56</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>platyphylloside</td>
<td>$r$</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td>$r$</td>
<td>-0.36</td>
<td>-0.37</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>proline</td>
<td>$r$</td>
<td>-0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.02</td>
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</tr>
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<td>aspartic acid</td>
<td>$r$</td>
<td>-0.39</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>essential amino acids</td>
<td>$r$</td>
<td>-0.37</td>
<td></td>
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<tr>
<td></td>
<td>$P$</td>
<td>0.05</td>
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$^a$Pearson’s correlation coefficient.
$^b$P-value of corresponding correlation above.
Discussion

This experiment is the first to demonstrate that predicted compositional changes of the troposphere could alter resistance of woody angiosperms to wood-boring insects. Throughout the four-year study period, elevated O$_3$ in combination with ambient CO$_2$ increased susceptibility of paper birch to bronze birch borer. However, elevated CO$_2$ ameliorated the negative affects of elevated O$_3$ on paper birch, as levels of bronze birch borer colonization were much lower in trees exposed to this treatment combination. This is consistent with a recent metaanalysis, which showed that folivore performance on plants exposed to elevated O$_3$ increased, but the positive effects of O$_3$ on folivore performance were negated by co-exposure to elevated CO$_2$ (Valkama et al. 2007).

Over time, levels of bronze birch borer colonization increased in trees exposed to elevated CO$_2$ in combination with ambient O$_3$. However, variation in the percent of trees colonized among rings was high and increased over time. It is unclear why substantial variation in levels of colonization was observed, but the dynamics could be quite different among rings, altering resistance of paper birch. For example, in some rings tall neighboring trees could be suppressing shade-intolerant paper birch, which would decrease rates of photosynthesis (Kubiske and Pregitzer 1996) and decrease the carbon pool available for allocation to secondary metabolites. Alternatively, some paper birch might allocate photosynthate primarily to growth, in order to maintain a dominant status in the canopy, which could also decrease the carbon pool available for allocation to
secondary metabolites if the source supply was limited. Both of these scenarios could result in more susceptible paper birch.

Results from this experiment are also consistent with the forest decline model proposed by Manion (1981). In Manion’s model, the combined impact of three successive and interactive stresses culminates in tree death (Manion 1981). Under this framework, O₃ could be considered a predisposing factor that kept paper birch in a long-term state of stress. Weakened, paper birch were less able to tolerate decreased rainfall from 2003-2006 (inciting factor) (Kubiske et al. 2006). In a feeble state, paper birch were vulnerable to colonization by bronze birch borer (contributing factor), to which they are otherwise resistant.

In an effort to explain the mechanism(s) underlying the patterns of bronze birch borer colonization, the chemistry of paper birch phloem was analyzed. Elevated CO₂ reduced nitrogen concentration in paper birch phloem, which corresponds with the general pattern of reduced nitrogen concentration of plants in enriched CO₂ environments (Drake et al. 1997). However, elevated CO₂ did not affect constitutive concentrations of free amino acids, indicating that the reduction occurred via reduced concentrations of stem proteins. In previous research, elevated CO₂ reduced nitrogen concentration in paper birch leaves (Lindroth et al. 2001, Mattson et al. 2005) and roots (Mattson et al. 2005), but not in stem phloem of seedlings (Mattson et al. 2005).

In this study, elevated CO₂ did not affect constitutive or induced concentrations of individual phenolics, total phenolics, or tannins in paper birch phloem. Defense theory predicts increased partitioning to secondary metabolites in plants exposed to elevated CO₂, if the net source supply (photosynthesis) is greater than sink demand (growth and
storage) during plant ontogeny (Herms and Mattson 1992, Mattson et al. 2005). In other studies of paper birch, elevated CO$_2$ increased allocation to phenolics or tannins in leaves (Mattson et al. 2005) and sapling stems (Mattson et al. 2004). However, previous research on paper birch at the Aspen FACE site indicated no change in concentrations of foliar tannins in response to elevated CO$_2$ (Kopper et al. 2001, Agrell et al. 2005). Why there was a deviation from the general trend of increased partitioning to phenolics in our study and others of paper birch at Aspen FACE is unclear. Previous research at Aspen FACE indicates an increase in net photosynthesis of paper birch (Riikonen et al. 2008), which would increase source capacity. However, carbon partitioning to secondary metabolites may be limited by sink strength as both increases in reproductive capacity (Darbah et al. 2008) and growth (data not shown) of paper birch also occurred under elevated CO$_2$.

Elevated O$_3$ decreased constitutive concentrations of one phenolic, betuloside, in 2007 and increased concentrations of betuloside in 2008. Elevated O$_3$ also decreased concentrations of two phenolics induced by bronze birch borer. However, elevated O$_3$ did not affect concentrations of the majority (83%) of individual phenolics, total phenolics, tannins, lignin, amino acids, or tree diameter. This was contrary to our prediction of ozone-induced stress and subsequent decrease in allocation to putative defenses due to disruption of photosynthesis. Therefore, if ozone is stressing paper birch as indicated by patterns of bronze birch borer colonization, it is operating by a mechanism that was not analyzed.

Elevated CO$_2$ in combination with ambient O$_3$, and elevated O$_3$ in combination with ambient CO$_2$, increased concentrations of three diarylheptanoids and decreased
concentrations of betuloside pentoside. However, similar to the lack of phytochemical responses in birch phloem to O$_3$, concentrations of 83% of individual phenolics as well as total phenolics, tannins, lignin, and amino acids were not affected by an interaction between CO$_2$ and O$_3$.

Induction treatments applied to phloem elicited both increases and decreases in concentrations of chemicals of paper birch. Induced concentrations of 94% of free amino acids were altered in response to induction treatments, of which 56% decreased and 38% increased. Induced concentrations of 42% of phenolics were impacted, of which 29% increased and 12% decreased, and induced concentration of lignin increased. In previous research, the same induction treatments increased concentrations of lignin, decreased concentrations of free amino acids, but did not affect concentrations of any phenolics in paper birch (Chapter 3). The consistent response for rapid increases in lignin concentrations indicates that lignin deposition could be an important part of a conserved, general plant response to physical injury. Lignified and suberized tissues are components of wound periderm, which isolate and protect injured tissues. The consistent, reduction of individual free amino acids to wounding also implicates their involvement in wound responses and may be used for synthesis of proteins involved in wound healing.

It is evident that CO$_2$ and O$_3$ fumigation altered paper birch phloem chemistry. However, do any of these changes correspond with patterns of bronze birch borer colonization among FACE treatments or can variation in phytochemistry explain the relationship between paper birch resistance and bronze birch borer colonization? At a coarse scale, variation in measured phytochemicals did not correspond with patterns of bronze birch borer among CO$_2$ and O$_3$ treatments. Unmeasured plant traits may underlie
observed patterns of colonization. Defensive proteins (Poyla 2003, Dafoe et al. 2009), water content (Hanks et al. 1999, Haukioja 2003), individual tannins (Barbehenn et al. 2009), host volatiles (Crook et al. 2008) and nutrients (Haack and Slansky 1987) have been implicated as important mediators of other plant-insect interactions and could contribute to paper birch resistance.

Quantitative variation of a few constitutive and induced phytochemicals was related to levels of bronze birch borer colonization, but many of these relationships were in the opposite direction than would be predicted. For example, concentrations of free amino acids, which putatively are nutritive, were inversely related with levels of bronze birch borer colonization, and concentrations of phenolics, which putatively are defensive, were positively related with levels of bronze borer colonization (Table 5.3). However, induced concentrations of two phenolics, procyanidin trimer and catechin, were inversely related to levels of bronze birch borer colonization and might be involved in resistance. Additionally, paper birch with medium to high levels of bronze borer colonization were characterized by high levels of lignin, carbon, and aspartic acid whereas trees with no colonization were characterized by high levels of phenylalanine, a precursor to synthesis of phenylpropanoids. Further research is needed to discern if these compounds contribute to paper birch resistance.

Concentrations of CO$_2$ are relatively homogenous over the earth, but concentrations of O$_3$ are variable as higher concentrations are localized to metropolitan areas and regions downwind to these areas (Tkacz et al. 2008). Therefore, it is plausible that the majority of paper birch trees, which largely exists in the boreal forests away from metropolitan areas, will experience an elevated CO$_2$ environment but not an elevated O$_3$
environment. As results from this work indicate, this could lead to increased
susceptibility of paper birch to bronze birch borer, and perhaps widespread mortality,
dramatically altering the composition and structure of North American boreal forests.
Moreover, it has been hypothesized that heat stress from global warming will increase
susceptibility of paper birch to bronze birch borer along the southern range of its
distribution, resulting in recession northward (Haack 1996). However, results from this
research indicate that elevated CO\textsubscript{2} could directly increase susceptibility of paper birch to
bronze birch borer on a range-wide basis, without a concomitant increase in temperature.

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References


ESRL/NOAA. 2010. Earth systems research laboratory (ESRL) / National oceanic and atmospheric administration (NOAA), Mauna Loa Observatory, Hawaii.


Chapter 6

Summary and suggestions for future work

Outbreaks by bronze birch borer (*Agrilus anxius*) have caused widespread birch (*Betula*) mortality, altering forest composition and possibly ecological trajectories (Balch and Prebble 1940, Jones et al. 1993). However, substantial variation in birch resistance to bronze birch borer exists due to genetic and environmental variability (Anderson 1944, Barter 1957, Miller et al. 1991, Nielsen et al. *in press*). *Betula* spp. endemic to North America, such as paper birch (*B. papyrifera* Marshall), share an evolutionary history with bronze birch borer and are highly resistant to successful colonization by larvae (Nielsen et al. *in press*). However, environmental stress has been associated with increased susceptibility of paper birch to bronze birch borer, leading to outbreaks and tree mortality (Balch and Prebble 1940, Jones et al. 1993). In contrast, tree species exotic to North America, such as European white birch (*B. pendula* Roth) do not share an evolutionary history with bronze birch borer and are much more susceptible (Ball and Simmons 1980, Miller et al. 1991, Nielsen et al. *in press*).
**Interspecific variation in Betula resistance to bronze birch borer**

The mechanism(s) underlying variation in resistance among *Betula* spp. to bronze birch borer are not understood. However, it is thought that both chemical and physical plant traits, of which many could be constitutively present or induced by larval feeding, contribute to woody angiosperm resistance to wood-boring insects (Eyles et al. 2007). In *Betula* spp., phenolics, including simple phenolics, tannins, and lignin, are dominant secondary metabolites that have been implicated as defenses in other birch-herbivore interactions (Julkunen-Tiitto et al. 1996, Ossipov et al. 1996, Kopper et al. 2002, Laitinen et al. 2004, Agrell et al. 2005, Mattson et al. 2005), but nutritive compounds may mitigate or augment the effectiveness of defenses (Slansky 1992, Simpson and Raubenheimer 2001, Haukioja 2003). In interactions between birch and bronze birch borer, previous research has implicated growth rate of wound-periderm (i.e. callus) tissue as important in birch resistance to bronze birch borer, which may function by physically encapsulating and killing feeding larvae (Anderson 1944, Miller et al. 1991).

This research showed that there is no difference between the rates of wound periderm formation between paper birch and European white birch. However, previous research found birch resistance to bronze birch borer to be correlated to wound periderm formation in paper birch but not in European white birch (Miller et al. 1991). Collectively, these findings suggest that if wound periderm contributes to paper birch resistance, then (1) the composition of wound periderm is different between species or (2)
wound periderm works in tandem with other plant attributes, such as a defensive metabolite.

In this research, comparison of the constitutive and induced chemical profiles of paper birch, which is much more resistant to bronze birch borer, and European white birch, which is much more susceptible, showed that they were chemically distinct (Fig. 6.1AB). Variation between species was due to qualitative and quantitative differences in phenolic composition. Two phenolics present in paper birch were not detected in European white birch and concentrations of six phenolics were higher in paper birch than in European white birch. These differences may contribute to resistance of paper birch to bronze birch borer. Notably, however, concentrations of total pools of phenolics and tannins do not correspond with interspecific differences in resistance as concentrations varied by year. In 2007, paper birch had higher concentrations of total phenolics than European white birch. Conversely, in 2008, European white birch had higher concentrations of total phenolics and tannins.

Experimentally applied bronze birch borer homogenate in combination with mechanical wounding did not elicit induced phytochemical responses that were different than induced by mechanical wounding alone. However, it is possible that paper birch, which shares a coevolutionary history with bronze birch borer, does elicit a specific, induced response to larval oral secretions and feeding. This hypothesis could be investigated by applying oral secretions from bronze birch borer in combination with a mechanical wound to paper birch or ideally, by caging mated females or applying eggs to paper birch and analyzing the natural induced responses to bronze birch borer.
Figure 6.1. Variation in A constitutive phenolics and B induced chemistry between paper birch (*Betula papyrifera*) and European white birch (*Betula pendula*).
However, in both paper birch and European white birch, mechanical wounding of stem phloem (with or without bronze birch borer homogenate) induced a chemical profile that was different than the constitutive profile (Fig. 3.3C). Differences between the induced and constitutive profiles were largely due to reductions in concentrations of free amino acids following wounding. The observed reductions may be due to increased demand for proteins necessary to synthesize compounds and tissues involved in general wound healing.

**The role of stress in paper birch-bronze birch borer interactions**

Tree stress, including that triggered by drought (Jones et al. 1993), high temperatures (Jones et al. 1993), advanced age (Balch and Prebble 1940) and experimental girdling (Anderson 1944, Barter 1957), has been associated with increased susceptibility of paper birch to bronze birch borer and can result in tree mortality. Girdling, specifically, alters host resistance by disrupting source-sink interactions within the tree (Anderson 1944, Christiansen and Ericsson 1986) via obstruction of phloem transport of current photosynthate from the leaves to portions of the tree below the girdle. This results in a depletion of photosynthate below the girdle and an accumulation of photosynthate above the girdle (Noel 1970, Goren et al. 2004). In paper birch, girdling increases susceptibility to bronze birch borer (Anderson 1944, Barter 1957), and within the tree, stem phloem below the girdle is more suitable for bronze birch borer larval development than stem phloem above the girdle (Anderson 1944). Because both susceptible (below girdle) and resistant (above girdle) tissue exits in a girdled tree,
girdling can be utilized as a tool to study tree resistance to wood-boring insects without confounding genetic and environmental variation.

Girdling increased susceptibility of paper birch to bronze birch borer colonization (Fig. 4.1), confirming that stress compromises resistance of paper birch. In girdled trees, there was also within-tree variation in levels of colonization as more bronze birch borer colonized stem phloem below than above the girdle (Fig. 4.1), supporting the hypothesis that the flux of current photosynthate from the leaves to the trunk is an important physiological mechanism underlying paper birch resistance. As local reserves diminish below the girdle, paper birch resistance may become dependent on the ability to mobilize carbohydrates necessary for biosynthesis of induced defenses at the site of damage or infestation (Christiansen and Ericsson 1986, Christiansen et al. 1987, Bonello et al. 2006).

Putative defensive mechanisms of paper birch were differentially affected above versus below the girdle. Girdling reduced concentrations of total phenolics and rate of wound periderm formation below the girdle in 1-year girdled trees. Girdling also reduced concentrations of induced total phenolics below the girdle over time, indicating increased carbon stress as phloem below the girdle continues to be isolated from the flow of current photosynthate. Additionally, levels of bronze birch borer colonization and induced phenolics were inversely related below the girdle, but there was no relationship above the girdle (Fig. 4.4). The effects of the girdling treatments on defensive mechanisms, and specifically, the a priori expectation for accumulation of photosynthate above the girdle, may have been obscured by preexisting and/or concurrent drought stress, as evidenced by very low rates of wound periderm formation and concentrations of phenolics, even in
ungirdled control trees. Drought stress reduces source strength (i.e. pool of available photosynthate) by decreasing stomatal conductance and photosynthesis (Flexas and Medrano 2002), which would dampen the effects of girdling on source-sink interactions.

**The effects of climate change on paper birch-bronze birch borer interactions**

In interactions between coevolved woody angiosperms and wood-boring insects, maintenance of tree vigor is important in bottom-up regulation of populations of wood-boring insects. Climate change may reduce vigor of woody angiosperms, increasing tree susceptibility to colonization by wood-boring insects, possibly provoking outbreaks. Elevated ozone ($O_3$), specifically, is detrimental to plants and has been implicated to predispose woody angiosperms to colonization by wood-boring beetles, leading to decline and mortality of trees (Manion 1981). Conversely, elevated carbon dioxide ($CO_2$) generally, but not always, increases carbon partitioning to secondary metabolites (Bezemer and Jones 1998, Koricheva et al. 1998), and therefore is expected to generally increase plant resistance to herbivores.

Results from the Free Air CO$_2$ Enrichment (Aspen FACE) experiment suggest that increases in concentrations of tropospheric CO$_2$ and O$_3$ could drastically alter resistance of paper birch to bronze birch borer, which could have important implications for the future distribution and abundance of paper birch. Alone, elevated CO$_2$ and elevated O$_3$ increased paper birch susceptibility to bronze birch borer (Fig. 5.1). In combination, however, elevated CO$_2$ and O$_3$ had no effect on paper birch resistance,
which is consistent with previous studies showing elevated CO$_2$ to ameliorate effects of elevated O$_3$ on tree physiology.

Results from the FACE experiment are also consistent with the decline model proposed by Manion (1981). In Manion’s model, the combined impact of three successive and interactive factors culminates in tree death (Manion 1981). Under this framework, elevated O$_3$ could be considered a predisposing factor that kept paper birch in a long-term state of stress. Weakened, paper birch were less able to tolerate decreased rainfall from 2003-2006 (inciting factor) (Kubiske et al. 2006), leading to vulnerability to colonization by bronze birch borer (contributing factor), which ultimately killed the trees.

Elevated CO$_2$ and O$_3$ altered phloem chemistry of paper birch, but not in ways that could explain patterns of bronze birch borer colonization. Elevated CO$_2$ decreased nitrogen content in phloem but did not affect concentrations of free amino acids, phenolics, tannins, or lignin. Elevated O$_3$ and the interaction between CO$_2$ and O$_3$ altered concentrations of three phenolics, but most phytochemicals were not affected by these treatments. Experimental wounding and bronze birch borer inoculation elicited induced phloem responses as increases in lignin concentrations and changes in concentrations of individual phenolics and amino acids were observed.

Additional implications of dissertation research

Collectively, my experiments indicate that interspecific variation in phytochemical composition may be more distinctive than variation in phytochemical composition generated by environmental stress. Phytochemical profiles of species more
resistant (paper birch) and more susceptible (European white birch) to bronze birch borer were clearly different (Fig. 6.1AB). This may be reflective of the stark variation in resistance and susceptibility of paper birch and European white birch: most bronze birch borer larvae cannot survive in healthy paper birch whereas most survive in European white birch (Miller et al. 1991, Nielsen et al. in press). In contrast, resistant and susceptible phenotypes of paper birch resulting from variation in stress could not be differentiated from one another by multivariate analysis of chemistry (Fig. 6.2AB). Perhaps this indicates that stress can produce a wide gradient of resistant phenotypes within paper birch, resulting in more subtle differences in phytochemistry. This would result in range of insect responses, from slightly reduced larval growth to larval mortality.

The experiments constituting this research indicate that there is no single variable that can explain paper birch resistance to bronze birch borer. Plant defense is generally complex, and I predict that the nature of birch defense to bronze birch borer will not be reduced to the presence or action of one chemical or plant trait. A complex, chemically mediated interaction between paper birch and bronze birch borer would be akin to the chemically mediated interaction between mountain birch (B. pubescens) and autumnal moth (Epirrita autumnata) (Haukioja 2003). Haukioja’s long-term work indicates that the effectiveness of birch defenses, as measured by autumnal moth larval growth, depends on the nutritive background as well as the amount of plant material consumed. Water content and protein content were particularly important in modulating the apparent effectiveness of phenolic defenses (Haukioja 2003). Haukioja also suggests that the effectiveness of phenolics as defenses may not be dependent on the concentrations of individual
Figure 6.2. Variation in A induced phenolics in girdled paper birch (*Betula papyrifera*) and B induced chemistry in paper birch exposed to ambient (low) and/or elevated (high) concentrations of carbon dioxide (CO$_2$) and ozone (O$_3$).
compounds but rather the total metabolic stress produced by several compounds eliciting the same insect detoxification mechanism.

Of the metabolites that were analyzed in this research, the most likely contributors to paper birch resistance were a few individual low molecular weight phenolics and total concentration of induced phenolics. However, because of their importance in other insect-plant interactions, future research investigating birch resistance to bronze birch borer should additionally analyze the role of other putative defenses such as defensive proteins (Poyla 2003, Dafoe et al. 2009) and individual tannins (Moilanen and Salminen 2008), as well as other putative nutrients such as proteins (Haukioja 2003), lipids (Behmer and Nes 2003), and water content (Hanks et al. 1999, Haukioja 2003). Likely, many plant traits, including some analyzed in this research, contribute to birch resistance, and the relative contribution of a certain metabolite to resistance will only be fully understood when considering larval growth and consumption and when juxtaposed against the metabolome. As this research indicates, genetic and environmental variation modifies the suite of metabolites present in birch, which may alter the effectiveness of individual defensive compounds on bronze birch borer performance.

An important insight this research provides is the potential impact that elevated CO$_2$ might have on future interactions between paper birch and bronze birch borer. Concentrations of CO$_2$ are relatively homogenous over Earth, but concentrations of O$_3$ are variable as higher concentrations are localized to metropolitan areas and regions downwind to these areas (Tkacz et al. 2008). Therefore, it is plausible that the majority of paper birch trees, which largely exists in the boreal forests away from metropolitan areas, will experience an elevated CO$_2$ environment but not an elevated O$_3$ environment. As
results from the FACE experiment indicate, this could lead to increased susceptibility of paper birch to bronze birch borer, and perhaps widespread mortality, drastically altering the composition and structure of North American boreal forests. Moreover, it has been hypothesized that heat stress from global warming will increase susceptibility of paper birch to bronze birch borer along the southern range of its distribution, resulting in recession northward (Haack 1996). However, results from this research indicate that elevated CO$_2$ could directly increase susceptibility of paper birch to bronze birch borer on a range-wide basis, without a concomitant increase in temperature. Finally, given that concentrations of CO$_2$ have already increased by 40% since the industrial revolution (Pearson and Palmer 2000, ESRL/NOAA 2010), elevated CO$_2$ may already be altering phenotypic resistance of paper birch to bronze birch borer. These changes may be occurring gradually, and therefore, have gone unnoticed. However, if a threshold of birch resistance is breached due to phenotypic alterations induced by elevated CO$_2$ and paper birch is less able to regulate population densities of this lethal herbivore, a rapid shift in the dynamics between paper birch and bronze birch borer may occur.
References


ESRL/NOAA. 2010. Earth systems research laboratory (ESRL) / National oceanic and atmospheric administration (NOAA), Mauna Loa Observatory, Hawaii


beech (Fagus sylvatica L.) grown under greenhouse conditions. Trees-Struct. Funct. 23: 539-553.


ESRL/NOAA. 2010. Earth systems research laboratory (ESRL) / National oceanic and atmospheric administration (NOAA), Mauna Loa Observatory, Hawaii.


Minitab Inc. 2007. Minitab 15 statistical software, State College, PA.


**Roslin, T., and J. P. Salminen. 2008.** Specialization pays off: contrasting effects of two types of tannins on oak specialist and generalist moth species. Oikos 117: 1560-1568.


White, T. C. R. 1993. The inadequate environment: nitrogen and the abundance of animals. Springer-Verlag, New York, New York, USA.


