Effects of poplar phenolics on the fitness and behaviour of *Chaitophorus* aphids

by

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BSc, University of British Columbia, 2007

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Supervisory Committee

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Abstract

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As sessile organisms, plants are unable to escape from attack by herbivorous insects. To cope with this pressure, plants have evolved several defense strategies, including the production of secondary metabolites, specialized chemicals with ecological functions. Most studies have focused on the role of secondary metabolites in plant defense against chewing insects. Little is known about what compounds are present in phloem sap and how they affect phloem feeding insects. Therefore, I investigated the effects of phenolic compounds on phloem feeders, using *Chaitophorus* aphids in bioassays with wildtype and transgenic poplar overexpressing the transcription factor MYB 134, which results in elevated levels of tannins and reduced levels of phenolic glycosides. Aphids produced significantly more offspring on MYB 134 plants but showed a significant preference for lower tannin leaf tissue. Analysis of poplar phloem exudates and aphid extracts provides direct evidence that the phenolic glycosides salicin, salicortin and tremulacin are present in poplar phloem and are ingested by aphids. These results are discussed in relation to what is driving the differences in aphid fecundity and choice between plant types.

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List of Abbreviations

ANOVA Analysis of Variance

CE Collision Energy

DP Declustering Potential

DW Dry Weight

EDTA Ethylenediaminetetraacetic acid

GLMM Generalized Linear Mixed Model

HPLC High Performance Liquid Chromatography

LC-MSMS Liquid Chromatography Tandem Mass Spectrometry

MRM Multiple Reaction Monitoring

NAD Nicotinamide Adenine Dinucleotide

PPO Polyphenol Oxidase

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Chapter 1. Plant defenses against insect herbivores

1.1 Insect feeding strategies

Plants are faced with many abiotic and biotic stresses. As primary producers that fix carbon via photosynthesis, plants are an energy source for many heterotrophic organisms (Mithöfer and Boland 2012). With over one million taxonomically diverse species, insect herbivores represent a major source of biotic pressure for plants (Howe and Jander 2008). Insect herbivores have many adaptations to use plants as a food source, including specialized feeding strategies. Insects can harvest plant tissue by chewing, mining, gall forming and sucking. Chewing insects are the most prevalent and use toothed mandibles to feed externally on leaf tissue (Strauss and Zangerl 2002). Leafmining insects feed on the tissue between leaf epidermal layers (Howe and Jander 2008). Gall forming insects manipulate their hosts to produce galls, which locally increase nutrients and provide shelter (Strauss and Zangerl 2002). Sucking insects in the order Hemiptera, such as aphids and whiteflies, use specialized mouthparts called a stylet to feed on phloem or xylem sap (Walling 2008). One of the major challenges plants face is that they are sessile organisms and unable to escape attack. The defenses plants have developed can be grouped into three categories, physical, chemical and associations with predators and parasites of herbivores. These are summarized below. Although this thesis focuses on plant defenses against aphids, most research on plant defense has been done with chewing insects. Therefore, this overview will primarily discuss defense against chewing insects.

1.2 Plant defenses against insect herbivores

1.2.1 Plant physical defense strategies against insects

The first lines of plant defense that insects encounter during feeding are physical structures or properties at the leaf surface such as trichomes, tough leaves and epicuticular waxes.

Trichomes

Trichomes are fine hair-like appendages that extend from the epidermis and show great diversity in size, shape and density (Levin 1973). They can be unicellular or multicelluar as well as non-glandular or glandular (Levin 1973). Non-glandular trichomes can serve as a barrier, preventing small insects from contacting the plant surface (Howe and Schaller 2008) whereas glandular trichomes secrete chemicals that interact with insect herbivores (Wagner 1991). The role of trichomes in plant defense is supported by the observation that insect feeding may induce their production. For example, whole *Salix ceneraea* (willow) plants showed an increase in trichome production when subject to *Phratora vulgatissima* (leaf beetle) feeding (Dalin and Bjorkmann 2003). This induction of trichomes serves to decrease herbivore damage by the subsequent generation of beetles. Second generation beetle larvae consumed less and displayed more dispersed feeding behaviour on plants that were previously exposed to beetles compared to second generation larvae feeding on undamaged plants (Dalin and Bjorkmann 2003).

Toughness

Leaf toughness is influenced by the lignin, cellulose, suberin and callose content in cell walls (Schoonhoven et al. 2005). It may protect against herbivory by decreasing the ease with which herbivores can break or penetrate the leaf or by influencing nutrient availability. Nutrient availability is affected by toughness because cell walls dilute the water and nutrient content of the leaf and are mostly indigestible by insects (Clissold et al. 2009). Evidence for leaf toughness protecting against herbivory was provided by a correlative study of 46 tropical tree species in which toughness was the best predictor of herbivory out of numerous leaf physical and chemical properties (Coley 1983). Further, a direct test of *Astrebla lappacea* (Mitchell grass) toughness on the performance of *Chortoicetes terminifera* (Australian plague locust) showed that growth rate was negatively affected by tough leaves as a result of reduced consumption, slower digestion and decreased nutrient assimilation (Clissold et al. 2009).

Epicuticular waxes

Epicuticular waxes are a layer of lipids that line the cuticle of most vascular plants and are extractable in relatively nonpolar organic solvents such as chloroform (Eigenbrode and Espelie 1995). These function primarily to prevent water loss but in some cases may provide the additional benefit of protecting against insect herbivores. The chemical composition of epicuticular waxes varies between and within species as well as with plant age and part (Eigenbrode and Espelie 1995). One way epicuticular waxes may protect against herbivory is by affecting insect attachment and movement. The psyllid species *Ctenarytaina spatulata* and *Glycaspis brimblecombei* made more stylet tracks to the vascular tissue when fed de-waxed leaves of *Eucalyptus globulus* than when fed waxy (untreated) leaves (Brennan and Weinbaum 2001). The number of vascular probes was positively correlated with survival and suggested to be due to increased feeding because of easier adhesion to the leaf surface (Brennan and Weinbaum 2001).

1.2.2 Plant chemical defenses against insects

The other type of defense strategy plants have evolved to protect against insect herbivores is the production of defense proteins or specialized chemicals called secondary metabolites. Defense proteins may have antinutritional properties or toxic effects (Mithöfer and Boland 2012). Secondary metabolites are compounds not required for normal plant growth or reproduction but have ecological functions (Howe and Jander 2008). Plants synthesize more than an estimated 200 000 secondary metabolites and these contain a great diversity of structures and modes of action (Mithöfer and Boland 2012). Some of the major classes of secondary metabolites include alkaloids, terpenoids, glucosinolates, cyanogenic glycosides and phenolics. Plant chemical defenses can be divided into two categories, those that are constitutive or always present and those that are induced in response to herbivory. Inducible defenses are activated upon recognition of an elicitor that can be of plant or insect origin (Howe and Jander 2008). The defense response activated is specific to the elicitor so that different insect herbivores can cause different plant responses (De Moraes et al. 1998). Plant defenses can also be classified as direct or indirect. Direct defenses act on their own to exert negative effects (Howe and

Jander 2008) whereas indirect defenses act by attracting enemies of their herbivores from a higher trophic level (Schnee et al. 2006).

Protein based defenses

Protease inhibitors

Protease inhibitors form complexes with proteases inhibiting their enzymatic activity and are one of the most common plant defense proteins. They can act against the insect in two ways. First, protease inhibitors may prevent the breakdown of other protein based defenses by insect digestive enzymes allowing them to exert their toxic or antinutritional effects. For example, the soybean cysteine protease inhibitor soyacystatin N prevents the breakdown of α-amylase inhibitor in wheat that is hypothesized to limit availability of simple carbohydrates (Amirhusin et al. 2004). *Callosobruchus maculates* (cowpea weevil) had slower development in an artificial seed diet containing both enzymes than when they were fed separately. When incubated with weevil gut extract, α-amylase was degraded but not if the gut extract was preincubated with soyacystatin N, supporting the hypothesis that soyacystatin N protects α-amylase inhibitor from being broken down by weevil digestive enzymes. Second, protease inhibitors may prevent the degradation of protein into amino acids thereby affecting nutrient availability for the insect (Ryan 1990). For example, *Manduca sexta* (tobacco hornworm) larvae that fed on transgenic *Nicotiana attenuata* (tobacco) plants with lower levels of trypsin proteinase inhibitor were heavier and had higher growth rates and survivorship (Zavala et al. 2004). In *N. attenuata*, the production of trypsin proteinase inhibitors is coordinated with the release of volatiles that attract the predator *Geocoris pallens* (big-eyed bug) that prefers eggs and young larvae (Zavala et al. 2004). The authors hypothesize that trypsin proteinase inhibtors act to slow the growth of larvae to keep them vulnerable to predation (Zavala et al. 2004).

Chitinases

Chitinases are enzymes that degrade chitin by hydrolyzing glycosidic bonds. Chitin is a component of the peritrophic membrane lining the insect midgut that separates

ingested food from the midgut epithelium (Barbehenn and Stannard, 2004). Chitinases may affect insect herbivores by disrupting this membrane (Howe and Jander 2008). *Leptinotarsa decemlineata* (Colorado potato beetle) had lower survival when fed leaves containing more than 0.3% w/w chitinase compared to controls (Lawrence and Novak 2006).

Lectins

Lectins are proteins that bind carbohydrates. They may affect insect herbivores by acting on their peritrophic membrane, digestive tract or glycosylated digestive enzymes (Peumans and Van Damme 1995). Lectins from several plant species have been shown to negatively affect the development of *C. maculates* (Peumans and Van Damme 1995). Lectins have been shown to affect phloem feeding insects as well. *Myzus persicae* (green peach aphid) had significantly lower fecundity and survival on transgenic *Nicotiana tabacam* (tobacco) that produced a mannose binding lectin compared to aphids feeding on controls (Kato et al. 2010).

Polyphenol oxidases

Polyphenol oxidases (PPO) are widely distributed copper containing oxidative enzymes. They have been suggested to have a role in herbivore defense because they are negatively correlated with *Heliothis zea* (tomato fruitworm) growth on tomato (Felton et al. 1989) and are induced by defense signaling molecules and a variety of herbivores (Constabel and Barbehenn 2008). PPOs produce quinones, which may result in antinutritive effects through the binding of protein or toxic effects through the production of oxidative stress (Constabel and Barbehenn 2008). Direct tests of PPO on *Malacosoma disstria* (forest tent caterpillar) and *Lymantria dispar* (gypsy moth) using transgenic poplar have demonstrated negative effects but only under certain seasonal conditions (Wang and Constabel 2004; Barbehenn et al. 2007). PPO activity requires oxygen and the low levels of oxygen in some insect guts (Johnson and Barbehenn 2000) may limit PPO activity. However, for plants that produce high levels of PPO like tomato, it is possible that it acts quickly pre-ingestion during tissue damage while insects are feeding (Howe and Jander 2008).

Secondary metabolites

Alkaloids

Alkaloids are nitrogen containing compounds and are common in the plant families Solanaceae, Papaveraceae, Apocynaceae and Ranunculaceae (Mithöfer and Boland 2012). The pyridine alkaloid nicotine is one of the most studied and its biosynthesis, transport and biological effects are well described. Nicotine is derived from putrescine and is synthesized in the roots of some Solanaceous plants, in particular members of the genus *Nicotiana* (Mithöfer and Boland 2012). It is toxic to many insects and its production is stimulated by herbivory. The negative effects of nicotine likely originate from its interaction with receptors of the neurotransmitter acytelcholine (Gepner et al. 1978), which are abundant in insects (Sattelle 1980). Upon herbivory, the signaling hormone jasmonate increases locally at the damage site and is transported to the roots where it activates nicotine synthesis (Mithöfer and Boland 2012). The newly synthesized nicotine is then transported via xylem sap to the leaves. When subject to mechanical wounding, *Nicotiana sylvestris* (tobacco) grown in the field increased alkaloid (nicotine and nornicotine) content in undamaged leaves (Baldwin 1988) compared to unwounded controls. When leaves from wounded and unwounded plants were fed to the specialist *M. sexta* in the lab, larvae gained less weight, consumed less and had poorer survival on the higher alkaloid leaves of wounded plants (Baldwin 1988). The role of nicotine in plant defense has also been directly tested through the use of transgenics. Silencing of the two putrescine N-methyl transferase genes in *Nicotiana attenuata* (tobacco) resulted in a 95% reduction in nicotine biosynthesis (Steppuhn et al. 2004). In a laboratory experiment with these plants, *M. sexta* had higher growth rates on and showed a preference for low nicotine leaves compared to wildtypes (Steppuhn et al. 2004). In the field, low nicotine plants suffered greater herbivory damage by the natural herbivores *Spodoptera exigua* (beet armyworm) and *Trimerotropis* grasshoppers (Steppuhn et al. 2004). However, some insects, such the tobacco hornworm, are tolerant to nicotine (Wink and Thiele 2002).

Terpenoids

Terpenoids (also called isoprenoids) are the most diverse class of secondary metabolites with more than 40 000 known structures (Howe and Jander 2008). They are carbon based compounds derived from 5-carbon isoprene units and are ubiquitous among plants (Mithöfer and Boland 2004). Lower molecular weight terpenoids including isoprene (C5), monoterpenes (C10) and sesquiterpenes (C15) make up most of the volatile terpenoids whereas higher weight terpenoids (C>15) are usually nonvolatile and are involved in cellular processes (Maffei et al. 2011). Volatile terpenoids can directly provide protection against herbivory by acting as a deterrent. When given a choice, tobacco hornworm larvae preferred control tobacco leaves over leaves that were genetically engineered to emit isoprene (Laothawornkitkul et al. 2008). Volatile terpenoids can also provide indirect defense by attracting predators or parasitoids. The parasitic wasp *Cotesia marginiventris* is attracted to (E)-β-farnesene, (E)-α-bergamotene and other sesquiterpenoids released by maize in response to Lepidopteran larval herbivory (Schnee et al. 2006). This has been demonstrated using olfactometer experiments. The maize terpene synthase gene TPS10 is responsible for the release of these damage-induced sesquiterpenes (Schnee et al. 2006). Female wasps preferred volatiles released by *Arabidopsis* expressing TPS10 over controls that did not produce sesquiterpenoids (Schnee et al. 2006).

Glucosinolates

Glucosinolates are sulfur containing compounds found almost exclusively in the order Capparales (Halkier and Gershenzon 2006). Their basic structure consists of a β-D-glucopyranose residue attached by a sulfur atom to a (Z)-N-hydroximinosulfate ester and an R group derived from an amino acid. Approximately 120 glucosinolates have been described and these can be classified into three groups based on the amino acids they are derived from. Aliphatic glucosinolates are derived from alanine, leucine, isoleucine, methionine or valine, aromatic glucosinolates are derived from phenylalanine or tyrosine and indole glucosinolates are derived from tryptophan. Glucosinolates are not typically harmful on their own, but upon tissue damage, they are hydrolyzed by separately stored myrosinases. The resulting product is unstable and rearranges to yield isothiocyanates,

nitriles, oxazolidine-2-thiones, epithionitriles and thiocyanates (Halkier and Gershenzon 2006; Textor and Gershenzon 2009). It is these hydrolysis products that are responsible for the biological activity of glucosinolates (Halkier and Gershenzon 2006). *Globodera rostochiensis* (potato cyst nematode) did not suffer mortality when exposed to glucosinolates alone, only when myrosinase was added (Buskov et al. 2002). The caddisflies *Hesperophylax designatus* and *Limnephilus sp.* preferred low phenylethyl glucosinolate and low nitrogen *Nasturtium officinale* (watercress) leaves over high phenylethyl glucosinolate and high nitrogen leaves (Newman et al. 1992). When leaves were heated to deactivate myrosinase to prevent the production of phenylethyl isothiocyanate, caddisflies preferred high glucosinolate leaves (Newman et al. 1992). Some glucosinolates can also break down during insect digestion in the absence of myrosinase and exert negative effects against phloem feeding insects (Kim et al. 2008).

Cyanogenic glycosides

Cyanogenic glycosides are derived from aliphatic and aromatic amino acids and are made up of an α-hydroxynitrile aglycone and a sugar moiety that is usually glucose (Zagrobelny et al. 2004). They are widely distributed among plants being found in more than 2500 plant species among several families including Fabaceae, Rosaceae, Linaceae, Compositae and others (Vetter 2000). They are a conjugated defense that is stored in the vacuole separately from the enzyme glucosidase in the cytoplasm. When cells are damaged by insect feeding, cyanogenic glycosides come in contact with glucosidase and generate toxic hydrogen cyanide in a two step process. First, glucosidase hydrolyzes the cyanogenic glycoside to form acetone cyanohydrin (Vetter 2000). Then, hydroxynitrile lyase converts acetone cyanohydrin to hydrogen cyanide (Vetter 2000). Hydrogen cyanide is toxic because it affects cellular respiration by preventing the binding of oxygen to cytochrome-c-oxidase in mitochondria (Mithöfer and Boland 2012). The breakdown of cyanogenic glycosides releasing hydrogen cyanide (cyanogenesis) appears to deter generalist insect herbivores like *Schistocerca gregaria* (desert locust) and *Spodoptera alittoralis* (cotton leafworm) but not specialists (Gleadow and Woodrow 2002).

Phenolics

Phenolics are a ubiquitous and diverse class of compounds that contain one or more hydroxyl group(s) directly attached to an aromatic ring. Although they are not broad spectrum defense compounds against insects (Treutter 2005), some phenolics such as hydroxycinnamic acids, flavonoids, furanocoumarins and tannins have been shown to have negative effects on insects.

Hydroxycinnamic acids

Chlorogenic acid is an ester of caffeic acid and quinic acid (Vermerris and Nicolson 2006) and has been shown to have negative effects on both chewing and piercing-sucking insects through its prooxidant activity. In *Lycopersicon esculentum* (tomato), chlorogenic acid is stored separately from PPOs but they react together to form chlorogenoquinone when tissue is damaged by chewing insects (Felton et al. 1989). *S. exigua* had significantly reduced growth rates when fed *L. esculentum* leaves without PPO inhibitors compared to leaves with PPO inhibitors (Felton et al. 1989). In the absence of PPO inhibitors, the oxidation product chlorogenoquinone produced in the insect gut was hypothesized to bind to amino acids and proteins and make them less digestible (Felton et al. 1989). However, the low oxygen environment of some insect guts (Johnson and Barbehenn 2000) may inhibit the activity of polyphenol oxidases, which require oxygen. The piercing-sucking insect *Frankliniella occidentalis* (western flower thrips) is also negatively affected by chlorogenic acid, having lower growth rates and survival on artificial diets containing 5% chlorogenic acid (Leiss et al. 2009). In addition, thrips preferred control diets without chlorogenic acid.

Flavonoids

Flavonoids are C15 compounds with a C6-C3-C6 skeleton with the arrangement of the C3 group determining their classification (Vermerris and Nicolson 2006). Some of these compounds have been shown to act as deterrents for insects in choice tests. First, when the flavone pectolinarigenin and the flavanone dihydrooroxylin A were isolated from *Nothofagus dombeyi* and *Nothofagus pumilio* (southern beech) respectively and added to artificial diets, *Ctenopsteustis obliquana* (brown leaf roller) showed a significant

preference for control diets without flavonoids (Thoison et al. 2004). Also, in choice tests, *Eurytides marcellus* (zebra swallowtail) butterflies laid fewer eggs on artificial plants painted with flavonoids even when the known oviposition stimulant, 3-caffeoyl-muco-quinic acid, was present (Haribal and Feeny 2003).

Furanocoumarins

Furanocoumarins are benz-2-pyrone compounds with a furan ring. The position of the furan ring determines whether they are linear (position 6,7) or angular (position 7,8) (Berenbaum 1981). Linear furanocoumarins are more widespread, having been reported in eight families, while angular furanocoumarins have only been reported in two (Berenbaum and Feeny 1981). Furanocoumarins are toxic to a wide variety of organisms including most insects (Chambers et al. 2007). For example, the linear furanocoumarin xanthotoxin is toxic through its ability to crosslink the strands of deoxyribonucleic acid (DNA) in the presence of ultraviolet light (Berenbaum 1978). This interferes with DNA replication and affects biological processes. When xanthotoxin was fed to *Spodoptera eridania* (southern armyworm) larvae in artificial diets, they did not survive past their second instar; however, larvae survived to pupation when fed the xanthotoxin precursor umbelliferone or if they were fed controls. All larvae produced fecal pellets so it was hypothesized that xanthotoxin interfered with development through toxicity and not starvation. Furanocoumarins are also induced by herbivory. For example, five different furanocoumarins were induced in *Pastinaca sativa* (wild parsnip) by herbivory by *Trichoplusia ni* (cabbage looper) (Zangerl 1990). This induction of furanocoumarins also negatively affects *T. ni* as caterpillars had lower growth rates on previously damaged leaves compared to undamaged leaves. Using artificial diets, it was found that one of the induced furanocoumarins, xanthotoxin, is likely responsible for this reduced growth (Zangerl 1990). These results suggest that xanthotoxin has both toxic and deterrent effects (Berenbaum 1981). Angular furanocoumarins are not toxic in the same way as linear furanocoumarins because the position of the furan ring is different (Berenbaum 1981). However, angular furanocoumarins have been shown to have negative effects on *Papilio polyxenes* (black swallowtail butterfly) growth and fecundity (Berenbaum and Feeny 1981).

Tannins

Tannins are widely distributed, high molecular weight polyphenolic compounds, that are defined by their ability to precipitate protein in vitro. They are classified into two major classes, the hydrolysable tannins and the condensed tannins. Hydrolysable tannins have a polyol core esterified with galloyl groups and include gallotannins and ellagitannins. Condensed tannins are oligomers or polymers of flavan-3-ols, usually catechin, epicatechin or trihydroxylated gallocatechins (Barbehenn and Constabel 2011). Tannins have long been hypothesized to play a role in plant defense since Feeny (1970) correlated Lepidopteran larvae damage on *Quercus robur* (common oak) with foliar tannin concentration. It was hypothesized that the protein binding ability of tannins led to decreased nutrient availability for the insect. However, subsequent studies showed that insect consumption is not always related to dietary tannin levels (Fox and Macauley 1977) and that protein digestibility is not affected by the addition of tannins to artificial diets in grasshoppers (Bernays et al. 1981). The basic pH of Lepidopteran guts may negate the putative antinutritive effects of tannins, as they are unable to bind protein above a pH of 9 (Fox and Macauley 1977). Alternatively, enzymes present in the insect gut may provide resistance to protein binding (Bernays et al. 1981). These findings suggest that tannins may have alternate modes of action such as prooxidant activity or deterrency. Tannins may be toxic through their oxidation that produces harmful oxygen radicals and quinones (Appel 1993). Clear evidence for the oxidation of tannins and their negative effects on insects has only been presented for hydrolysable tannins (Barbehenn et al. 2009). High levels of gut antioxidants have been attributed to the hydrolysable tannin tolerance of *Orgyia leucostigma* (white marked tussock moth) (Barbehenn et al. 2001). Tannins may also act as deterrents due to their astringent taste (Feeny 1970; Appel 1993). Studies have reported tannins having stimulatory (Barbehenn and Constabel 2011), deterrent (Bernays et al. 1981; Manuwoto and Scriber 1986) and no (Osier et al. 2000; Kosonen et al. 2012) effects on insect feeding. Thus, the evidence for tannins in plant defense against insect herbivores is mixed. It should be noted that most of the research has focused on the effects of tannins on chewing insects; little is known about

how phloem feeders are affected. This is the area that this thesis aims to address (see below).

1.2.3 Plants can use third trophic level associations for indirect defense against insect herbivores

Some plants benefit from the indirect defense provided by the predators or parasitoids of their herbivores. Many plants, termed myrmecophytes, have mutualistic relationships with ants that attack approaching insect herbivores in return for housing. For example, *Acacia cornigera* (swollen thorn acacia) has a mutualistic relationship with *Pseudomyrmex ferruginea* that protects it by attacking insects that contact the plant surface (Janzen 1967). In return, the plant provides ants with housing in its stipular thorns and nutrients in the form of nectar and Beltian bodies at the tips of its leaflets. When ants are removed, *A. cornigera* suffers greater herbivory damage and eventually death as it has lost deterrent mechanisms that other *Acacia* species have. Another example of this plant-ant mutualism is *Tachigali myrmecophilia* that provides housing for *Pseudomyrmex concolor* that protects it from insects. When ants were removed, *T. myrmecophilia* suffered ten times more damage than plants with ants (Fonseca 1994).

Some plants release volatiles in response to herbivory that parasitoids use to locate their hosts. For example, flight assays showed that the parasitoid *Cotesia marginiventris* is attracted to the volatiles released by corn seedlings in response to feeding by *S. exigua* (Turlings et al. 1990). Plants may even release volatiles in response to specific herbivores that parasitoids are able to distinguish. In the field, the parasitoid *Cardiochiles nigriceps* is able to distinguish between volatiles emitted by tobacco, cotton and maize in response to its host *Heliothis virescens* (tobacco budworm) and it nonhost *Helicoverpa zea* (cotton bollworm, corn ear worm, tomato fruitworm) (De Moraes et al. 1998). Tobacco and maize release different quantities of specific volatiles while cotton releases different types of volatiles in response to the different caterpillars (De Moraes et al. 1998).

1.3 Insect strategies against plant defense compounds

Insect herbivores have evolved diverse strategies to overcome these sophisticated physical and chemical plant defenses. Insects cope with plant defense chemicals behaviourally by avoiding or deactivating them. They also deal with defense compounds post ingestion by detoxifying or conjugating them to decrease their toxicity. Some insects may possess mutations that prevent defense compounds from binding to their receptors therefore negating their effects. This is referred to as target site insensitivity.

1.3.1 Behavioural strategies

Avoidance

One behavioural strategy that insects use to cope with plant defenses is avoidance. This may be innate or learned. *Trichoplusia ni* larvae selectively eat around the veins of wild parsnip that contain furanocoumarins (Karban and Agrawal 2002). Also, cotton leaf perforator larvae have been observed to avoid eating the epidermis and pigment of wild cotton that contains terpenoid aldehydes (Karban and Agrawal 2002). Learning has been demonstrated with grasshoppers. For example, in a study by Bernays and Lee (1988), two groups of the polyphagous grasshopper *Schistocerca americana* were given an adverse stimulus prior to being fed either spinach or broccoli. Both groups were then fed spinach. The grasshoppers that were fed spinach with the adverse stimulus ate less spinach later compared to those that ate broccoli with the adverse stimulus (Bernays and Lee 1988).

Defense deactivation

Another behavioural tactic that insects use to deal with plant defenses is to deactivate them. Defense deactivation is employed for compounds stored under pressure in latex ducts or resin canals and is achieved by severing these canals allowing the defensive fluid (latex or resin) to spill out, and then feeding distally. *Asclepias syriaca* (milkweed) contains latex stored under pressure in laticifers that run along the veins of leaves. This latex is a feeding deterrent to insects and also coagulates with air exposure, hardening to form a muzzle around the insect's mouth (Dussourd and Eisner 1987). When three specialist insects on milkweeds, *Labidomera clivicollis* (chrysomelid beetle),

Tetraopes tetrophthalmus (cerambycid beetle) and *Danaus plexippus* (monarch butterfly) and three generalist insects *Popillia japonica* (Japanese beetle), *Pyrrharctia isabella* (woolly bear caterpillar) and *S. eridania* were placed on an intact milkweed leaf and then onto a treatment milkweed leaf that had simulated vein cutting on one side, all insects fed readily on the cut side of the treatment leaf (Dussourd and Eisner 1987). Only the specialists, however, fed on the control and uncut side of the treatment leaf, severing veins and feeding away from the cut. Another example of a specialist using this strategy is the beetle *Blepharida sp*. that feeds on the deciduous shrub *Bursera schlechtendalii* by cutting canals to release resin that normally squirts out upon damage (Becerra 1994). This cutting behaviour is time intensive compared to the time it takes to feed on the disarmed leaf. Some generalist insects such as *T. ni* are able to cut veins in order to feed on plant species that exude secretions (Dussourd and Denno 1994). Other generalists like *Spodoptera ornithogalli* (yellow-striped armyworm), however, are unable to cut veins and perform poorly on plants with resin and latex but do well on deactivated leaves.

1.3.2 Post ingestion strategies

Insect herbivores also deal with plant defenses post ingestion by producing enzymes that are involved in detoxification or compounds that render defensive compounds harmless. Several strategies including detoxification and conjugation have been described. Some insects may also have mutations that prevent defense compounds from binding to their receptors.

Detoxification

Insect herbivores cope with ingested defense compounds by converting them into less toxic forms. For example, the specialist *Pieris rapae* (small white butterfly) produces a gut nitrile-specifier protein that redirects the hydrolysis reaction between glucosinolates and myrosinase towards nitrile rather than isothiocyanate production (Wittstock et al. 2004). Nitriles are less toxic than isothiocyanates and are excreted in the feces by the butterfly, either unmodified or after further metabolism. The prevention of isothiocyanate production appears to be the mechanism by which *P. rapae* can feed on plants containing glucosinolates.

Conjugation

Another way that insect herbivores deal with plant defense compounds post ingestion is by conjugation. In this strategy the insect produces a compound that binds with harmful compounds in a way that renders them harmless (Strauss and Zangerl 2002). An example of this is the specialist *Plutella xylostella* (diamondback moth) that is able to feed on cruciferous plants despite their production of glucosinolates. This species produces a gut glucosinolate sulfatase that protects it from the glucosinolate-myrosinase system in two ways (Ratzka et al. 2002). First, it desulfates glucosinolates producing desulfoglucosinolates that are unable to be used as a substrate by myrosinase. This prevents the production of toxic breakdown products. Second, the sulfatase competes with myrosinase for glucosinolate as a substrate. The sulfatase produced by *P. xylostella* acts on different classes of glucosinolates allowing the moth to use a broad range of cruciferous plants as hosts.

Target site insensitivity

Some insects have mutations that prevent toxic defense compounds from binding to their specific receptors. This type of resistance is called target site insensitivity. For example, some strains of *P. xylostella* are resistant to pyrethroids, synthetic compounds similar to the terpenoid pyrethrins naturally produced by *Chrysanthemum*. Pyrethroids bind to sodium channel receptor proteins in insects disabling their nervous system and causing paralysis (Strauss and Zangerl 2002). Two amino acid substitutions within the sodium channel likely confer resistance in these moth strains by reducing binding of pyrethroids (Schuler et al. 1998). One of these mutations (leucine to phenylalanine) has also been correlated with resistance in *Musca domestica* (house fly) and *Blattella germanica* (German cockroach) (Schuler et al. 1998). Another example of target site insensitivity is *D. plexippus* that is able to feed on milkweed plants that produce cardiac glycosides as well as sequester them for their own defense against predators (Holzinger and Wink 1996). Cardiac glycosides target the enzyme sodium and potassium ATPase that maintains the balance of sodium and potassium across cell membranes. This binding disrupts the ion gradient necessary for cellular processes. The monarch butterfly has a

single amino acid substitution (asparagine to histidine) on the binding site of the cardiac glycoside ouabain (Holzinger and Wink 1996). When the same mutation was introduced to the ouabain binding site of *Drosophila*, flies suffered less mortality than untransformed flies. This confirmed that the monarch butterfly ATPase substitution is responsible for its resistance. This substitution may be what allows *D. plexippus* to exploit cardiac glycosides.

1.4 Role of plant secondary metabolites in defense against phloem feeding insects

A great amount of progress has been made in understanding the effects of secondary metabolites on insect herbivores; however, most of the work has focused on chewing insects and relatively little is known about the effects on phloem feeders. Phloem feeding insects in the order Hemiptera feed on phloem sap within sieve tube elements using specialized mouthparts collectively called a stylet. It was once believed that insects like aphids that use phloem sap as their dominant or sole food source avoided plant secondary metabolites stored in the vacuole of mesophyll cells (Peng and Miles 1991). This idea suggests that plant resistance should be related only to characteristics of the phloem such as nutritional quality or presence of defense chemicals. More recently it has been recognized that plant chemicals in the mesophyll do influence host plant acceptance. For aphids, the length of time probing before reaching and then ingesting phloem is related to host plant resistance (Dreyer and Campbell 1987). Aphids spend a longer time probing and shorter time ingesting on resistant plants. It has also been shown that aphid host plant acceptance is related to the probing of mesophyll cells along the stylet path before contact with the phloem (Tosh et al. 2002). Therefore, the role of secondary metabolites in plant defense against phloem feeders warrants more attention. There is substantial evidence that at least some secondary plant metabolites are found in the phloem itself. For example, *Arabidopsis thaliana* and *Manihot esculenta* (cassava) contain glucosinolates (Chen et al. 2001) and cyanogenic glycosides (Jorgensen et al. 2005) respectively. In *Arabidopsis* it has been shown that secondary metabolites affect phloem feeding insects (Kim et al. 2008).

1.5 Phloem feeding insects

Phloem feeding insects use specialized mouth parts called a stylet to feed on plant phloem sap within sieve tube elements (Walling 2008). Animals that use phloem sap as their main or only food source are restricted to the order Hemiptera (Douglas 2006). These include aphids, whiteflies, mealybugs and psyllids in the suborder Sternorrhyncha, planthoppers and leafhoppers in the suborder Auchenorrhyncha and most plant feeders in the suborder Heteroptera (Douglas 2006).

1.5.1 Phloem feeders as agricultural pests

Phloem feeding insects are major pests on agricultural crops (Powell et al 2006; Thompson and Goggin 2006). For aphids, the ability to reproduce at high rates and disperse under unfavorable conditions contributes to their success as pests. Aphid life history typically involves multiple generations of asexual reproduction and one generation of sexual reproduction (Moran 1992). In the asexual mode of reproduction, aphids reproduce by parthenogenesis in which females give birth to genetically identical live young. Parthenogenesis can give rise to wingless or winged morphs depending on the environmental conditions (Brisson 2010). The production of winged morphs can be in response to high density or poor plant quality, which allows the aphid to disperse more easily and find a new suitable host plant. In addition to causing crop damage, phloem feeding insects are also vectors of plant diseases (Thompson and Goggin 2006). Therefore, it is important to understand how plants defend against phloem feeders.

1.5.2 Feeding processes and strategies of phloem feeders

Although all phloem feeders use a stylet to feed on phloem sap, they differ in their feeding tactics, stylet paths, use of sheath saliva and composition of watery saliva (Kaloshian and Walling 2005). These differences affect their contact with and induction

of plant defenses and should be kept in mind when considering the plant defense response to different types of phloem feeders.

Feeding tactics and stylet path

The stylet paths of aphids (family Aphididae) and whiteflies (family Aleyrodidae) both contain multiple branches (Walling 2008). While aphids puncture and "taste" most cells along the stylet path, whiteflies rarely puncture mesophyll cells. In addition, aphids use more than one feeding site during their life whereas whiteflies feed continuously at a single site during their development, only retracting their stylet during molts (Kaloshian and Walling 2005; Walling 2008).

Saliva

Phloem feeding insects secrete two types of saliva that facilitate their feeding, sheath saliva and watery saliva. Sheath saliva is secreted at the surface of the leaf and then continuously in beads along the stylet path (Tjallingii 2005; Walling 2008). This saliva acts to prevent the stylet from slipping at the leaf surface and to form a barrier between the stylet and plant host tissue protecting it from apoplastic defenses (Kaloshian and Walling 2005). It also seals the puncture site of the sieve tube element preventing the plant from responding to the damage by producing callose to plug the hole (Will and van Bel 2006). Sheath saliva is mostly made up of proteins, phospholipids and conjugated carbohydrates and is not secreted by all phloem feeding insects, for example mirids (Miridae) (Kaloshian and Walling 2005). Watery saliva is secreted along the stylet path during intracellular probing as well as before and during phloem sap ingestion (Tjallingii 2005; Will et al. 2012). It is involved in tasting mesophyll cell contents along the stylet path and preventing the occlusion of sieve tube elements before and during ingestion. Some common salivary proteins include phenoloxidases, peroxidases, pectinases, amylases, alkaline and acidic phosphatases, proteases and lipases (Miles 1999). These enzymes have been hypothesized to maintain pH conditions, detoxify phenolics, loosen cell walls or prevent the plugging of sieve tube elements (Miles 1999) but direct evidence for these functions is somewhat lacking.

1.6 Phloem

1.6.1 Phloem physiology

In vascular plants, phloem sap is the transport system for nutrients, some defense compounds and information signals (Turgeon and Wolf 2009). Phloem sap is located within contiguous cells called sieve tube elements that are associated with companion cells connected via plasmodesmata pore units. Together these two cells form a functional phloem complex. The phloem complex is connected to surrounding parenchyma and other cells by plasmodesmata that are primarily connect with the companion cells. Transport of molecules into the complex is determined by the abundance and permeability of plasmodesmata. Plasmodesmata transport is passive for small molecules in the cytosol and limited by size, conductivity of the pore and possibly charge. For molecules in the apoplast, transport across the plasma membrane can be passive, transporter driven or endocytic.

1.6.2 Sampling phloem sap

Phloem sap is difficult to sample. Sieve tube elements are under high hydrostatic pressure and any perturbation leads to rapid occlusion of the sieve elements at the sieve plates. Some methods developed to sample phloem sap include stylectomy (Fisher and Frame 1984), bleeding (Pate and Sharkey1974), ethylenediaminetetraacetic acid (EDTA) facilitated exudation (King and Zeevaart 1974) and isotopic labeling (Chen et al. 2001). Although several methods for sampling phloem sap have been developed, no method to date provides a complete and accurate picture of what is contained in phloem (Turgeon and Wolf 2009). The purity of phloem sap is assessed using the expected molecular profile of phloem sap, which is high concentrations of transport carbohydrate, high molar ratio of sugars to amino acids and little or no monosaccharides (Turgeon and Wolf 2009). Typically, the absence of monosaccharides is an indicator of purity (Turgeon and Wolf 2009).

1.6.3 Phloem contents

Despite the challenges in sampling phloem sap, sugars, amino acids, secondary metabolites, proteins and possibly RNA have been shown to be present in phloem. I will focus on the secondary metabolites. There is evidence that glucosinolates, quinolizidine alkaloids, cyanogenic glycosides, pyrrolizidine alkaloids and terpenoids are present in phloem sap of different plant species. Some of these compounds have been tested for biological effects on phloem feeders, and these will be reviewed later.

1.6.4 Phloem sap as a food source

From the perspective of insect herbivores, phloem is a nutritionally imbalanced food source containing high levels of sugar and low levels of essential amino acids (Thompson and Goggin 2006). This creates two problems for phloem feeding insects - meeting their nutritional requirements and coping with the high osmotic pressure of phloem sap (Douglas 2006).

Like all animals, phloem feeding insects cannot synthesize nine of the amino acids necessary for their growth and reproduction and must obtain these from their food source; however, phloem sap is deficient in essential amino acids (Douglas 2006). All phloem feeding insects have symbiotic microorganisms that provide their hosts with these essential amino acids missing in their diet (Douglas 2006). The best evidence for this is for aphids which have *Buchnera sp*., a vertically transmitted obligate bacterial symbiont (Oliver et al. 2010). Although *Buchnera* has a very small genome, it has retained the genes coding for the biosynthetic enzymes for essential amino acids (Douglas 2006). This strongly suggests that aphids have overcome the problem of poor nitrogen quality in phloem through their association with *Buchnera*.

The primary component of phloem sap is sucrose, which results in high osmotic pressure. The osmotic pressure in the gut of phloem feeding insects predicts that they should shrivel up due to the movement of water from body fluids into the gut (Douglas 2006). Although only demonstrated for aphids, phloem feeders may convert sucrose to oligosaccharides to be excreted as honeydew, which reduces gut osmotic pressure (Douglas 2006).

1.7 Plant defenses against phloem feeders

In contrast to defense against chewing insects, little is known about what factors are important for defense against phloem feeders (Walling 2000; Zhu-Salzman et al. 2004). However, plant molecular responses to a few phloem feeders have been analyzed and may provide clues about resistance mechanisms. Studies have shown that plants respond to phloem feeding insects with an induction of signaling compounds involved in defense (Thompson and Goggin 2006) and pathogenesis related proteins (Walling 2000). Although these studies suggest a role for these responses in defense against phloem feeders, their specific functions remain unknown. I will focus here on the defenses known to be effective against phloem feeders by reviewing studies that have directly tested for an effect of signaling hormones, resistance genes and secondary metabolites on phloem feeder fecundity, development or behaviour.

1.7.1 Damage mediated plant defenses

Jasmonic Acid

Jasmonic acid is an oxygenated lipid plant hormone derived from the fatty acid linolenic acid and is released from plastid membranes in response to all types of herbivore feeding (Kaloshian and Walling 2005). This signaling molecule regulates genes involved in defense (Zhu-Salzman et al. 2004). The direct role of jasmonic acid mediated defenses in plant defense against phloem feeding insects has been investigated using plants treated with methyl jasmonate and mutants. For example, *Schizaphis graminum* (greenbug) preferred control *Sorghum bicolour* (sorghum) seedlings over ones that were treated with methyl jasmonate (Zhu-Salzman et al. 2004). Also, *M. persicae* fecundity was higher (Ellis et al. 2002) and *Bemisia tabaci* type B (silverleaf whitefly) development was faster (Zarate et al. 2007) on mutants of *Arabidopsis* that were insensitive to jasmonic acid (coi1). These studies suggest that jasmonic acid induced defense is involved in defense against phloem feeders, as it is against chewing insects (Howe and Jander 2008).

Salicylic Acid

Although salicylic acid is induced by phloem feeding insects, direct tests of salicylic acid induced defense on phloem feeders using *Arabidopsis* mutants suggest that it does not play a direct role in defense (Thompson and Goggin 2006). For example, there was no difference in *M. persicae* fecundity when aphids were grown on mutants with reduced salicylic acid accumulation (eds5) compared to on wildtype plants (Moran and Thompson 2001). Salicylic acid accumulation may even benefit phloem feeding insects. For example, *B. tabaci* development was slower and *M. persicae* and *B. brassicaceae* had reduced performance on mutants with reduced salicylic acid accumulation (NahG) (Mewis et al. 2005; Zarate et al. 2007). Caution should be taken when interpreting these results though as the effects of NahG are not limited to salicylic acid (Thompson and Goggin 2006).

1.7.2 Plant resistance genes

Single genes conferring resistance to phloem feeders have been identified. For example, the Meu-1 gene in tomato affects plant resistance to specific biotypes of *Macrosiphum euphorbiae* (potato aphid). Using isogenic lines that differed in alleles of Meu-1, it was shown that aphids fed less and had significantly lower fecundity and survival on the resistant line (Kaloshian et al. 1997). This Meu-1 gene was later confirmed to be the same as Mi-1 in tomato (Rossi et al. 1998) and also shown to confer resistance to *B. tabaci* (Nombela et al. 2003) and *Bactericerca cockerelli* (psyllid) (Casteel et al. 2006). It appears that resistance genes have a narrow efficacy against phloem feeders as they often confer resistance to a single or small number of aphid biotypes (Walling 2000). For example, Mi-1 was effective against one of two biotypes of *M. euphorbiae* tested (Rossi et al. 1998) and the Sd1 gene in apple provided resistance to two of three biotypes of *Dysaphis devecta* (rosy leaf curling aphid) tested (Roche et al. 1997).

1.7.3 Secondary metabolites

The role of secondary metabolites in plant defense against phloem feeders has received less attention than for chewing insects. Still, some secondary metabolites have been shown to be present in phloem sap and have been investigated for their effects on phloem feeders. These include glucosinolates and quinolizidine alkaloids and cyanogenic glycosides.

Quinolizidine alkaloids

Quinolizidine alkaloids are derived from the amino acid lysine, contain a quinolizidine ring or a piperidine ring and are present mostly in the family Leguminosae (Bunsupa et al. 2012). They are synthesized mainly in the shoots (Bunsupa et al. 2012) and transported in the phloem of *Lupinus* species to seeds and mature fruit where they accumulate (Wink and Witte 1984; Lee et al. 2007). Quinolizidine alkaloids are ingested by aphids and affect host selection. The generalist *Acyrthosiphon pisum* (pea aphid) was deterred by high levels of quinolizidine alkaloids in *Cytisus scoparius* (broom) (Wink et al. 1992). However, some aphids like the specialist *Aphis cytisorum* (broom aphid) feed on broom plants with intermediate levels of quinolizidine alkaloids and accumulate them in their bodies, possibly to protect against predators by making themselves less palatable (Wink and Witte 1985; Wink et al. 1992).

Glucosinolates

As mentioned previously, glucosinolates are conjugated defense metabolites that interact with myrosinases that are stored separately to form deterrent isothiocyanates, nitriles and thiocyanates. Glucosinolates are transported in the phloem sap of *A. thaliana* (Chen et al. 2001) and phloem feeding insects are able to feed without bringing them in contact with activating myrosinases. The indole glucosinolate indol-3-methylglucosinolate (IM3) however, is still effective against *M. persicae* as its digestion yields breakdown products that react to form harmful compounds with antifeedant effects (Kim et al. 2008). Aphids had significantly lower fecundity on *Arabidopsis* mutants with high IM3 than wildtype plants (Kim et al. 2008). Interestingly, some phloem feeders are able to use glucosinolates for their own benefit. *Brevicoryne brassicae* (cabbage aphid)

sequesters glucosinolates in its hemolymph and also synthesizes its own myrosinase enzyme (Kazana et al. 2007).

Cyanogenic glycosides

Cyanogenic glycosides are widespread conjugated defenses that react with separately stored glucosidase to produce hydrogen cyanide upon cell damage (Vetter 2000). Indirect evidence suggests that cyanogenic glycosides are present in phloem sap. First, unidentified cyanogenic glucosides were found in phloem secretions of *Manihot esculenta* (Calatayud et al. 1994). Cyanogenic glucosides and free cyanides were also found in the honeydew of *Phenacoccus manihoti* (mealybug) grown on *M. esculenta* (Calatayud et al. 1994). Second, when phloem tissue was removed to prevent the movement of phloem-transported molecules, cyanide levels were higher above the incision, supporting the hypothesis that cyanogenic glucosides are transported in phloem from leaves to roots (Jorgensen et al. 2005).

Little is known about how cyanogenic glycosides affect phloem feeding insects. Mealybug infestation did not increase cyanide levels and free cyanide content did not correlate with resistance in several genotypes of *M. esculenta* (Calatayud et al. 1994). When added to mealybug artificial diet, the cyanogenic glycoside linamarin did not affect the growth or development time of *P. manihoti* (Calatayud 2000).

1.8 Introduction to the experimental system and research objectives

1.8.1 Poplar as a system to study plant defense against insect herbivores

Phenolics in poplars

Populus (poplar) is a widely distributed genus in the Northern Hemisphere consisting of approximately 30 species. As long lived tree species, poplars must deal with insect herbivores that may go through many generations within the tree's lifetime. Poplar produces a variety of phenolic secondary metabolites of which condensed tannins and salicin based phenolic glycosides are generally the most abundant in leaves (Lindroth and

Hwang 1996). Both tannins and phenolic glycosides have been hypothesized to protect against chewing insects with studies showing mixed effects for tannins (Barbehenn and Constabel 2011) and strong effects for phenolic glycosides (Boeckler et al. 2011). There is a lack of studies investigating whether these phenolics affect phloem feeding insects. (Boeckler et al. 2011; Barbehenn and Constabel 2011)

Condensed tannins in poplar

Poplars produce condensed tannins and their abundance is influenced by many factors, including genetics. Different genotypes of *Populus tremuloides* have been reported to have between 2% and 25% of their dry leaf weight made up of tannins when grown under the same conditions (Hwang and Lindroth 1997). Ontogeny also influences tannin levels with mature leaves accumulating more than twice as much compared to developing leaves (Donaldson et al. 2006). Finally, environmental factors such as nutrient availability, ultraviolet radiation, and light exposure can also affect tannin levels (Hemming and Lindroth 1999; Mellway et al. 2009).

Tannins are induced in *P. tremuloides* by *M. disstria* and *Lecoma salicis* (satin moth) larvae herbivory (Peters and Constabel 2002) suggesting that they are involved in plant defense. However, studies that have investigated the effects of tannins on chewing insects have yielded mixed results suggesting that tannins are not broad antiherbivore compounds (Ayres et al. 1997). Much of the research on the effects of tannins in poplar has focused on chewing insects. It is not known if tannins or their monomers catechin or epicatechin are present in poplar phloem sap and if they affect phloem feeders.

Phenolic glycosides in poplar

Broadly speaking, a phenolic glycoside is any molecule with a sugar bonded to a phenol aglycone. Salicin based phenolic glycosides or “salicinoids” are unique to the family Salicaceae (of which poplar are members) and consist of approximately 20 compounds (Boeckler et al. 2011). From here on, I will use the term phenolic glycosides to refer specifically to salicin based phenolic glycosides. Phenolic glycosides have a core made up of salicyl alcohol and a β-d-glucopyranose, with an ether linkage between the phenolic hydroxyl group and the anomeric carbon atom of the glucose. The simplest

phenolic glycoside is salicin with more complex phenolic glycosides having organic acids esterified to one or more hydroxyl groups. Phenolic glycosides may also have gentisyl alcohol instead of salicyl alcohol as their basic aglycone. The most commonly reported phenolic glycosides in *Populus spp.* are salicin, salicortin, tremulacin and tremuloidin (Boeckler et al. 2011).

The abundance of phenolic glycosides within Salicaceae is primarily determined by genetics (Boeckler et al. 2011) with species like *P. tremuloides* having up to 30% of its leaf dry weight made up of phenolic glycosides (Donaldson et al. 2006), while other species do not accumulate detectable levels (Palo 1984). The abundance of phenolic glycosides is also dependent on ontogeny with juvenile tissues having the highest levels. Foliar phenolic glycosides decrease exponentially with plant age in *P. tremuloides* (Donaldson et al. 2006). Within a shoot, leaf age is negatively correlated with phenolic glycoside abundance (Kleiner et al. 2003). Abiotic factors such as resource availability also influence phenolic glycoside abundance (reviewed in Boeckler et al. 2011).

Although they have been shown to have negative effects on chewing insect herbivores (Hwang and Lindroth 1997; Osier et al. 2000), phenolic glycosides are not rapidly induced by herbivore damage (Stevens and Lindroth 2005). No studies have directly tested how phenolic glycosides affect phloem feeding insects. In addition, it is not well known whether phenolic glycosides are present in phloem sap. Only a single study has reported salicin in the phloem exudates of *P. deltoides* (Gould et al. 2007).

1.8.2 Genetic transformation and manipulation of phenolic synthesis in poplar

Because poplar produces a variety of phenolic compounds and the levels of these can vary between and within poplar species (Hemming and Lindroth 1995), it can be challenging to relate plant resistance to herbivory to a particular compound. Using genetic transformation to produce plants altered in a specific compound is a useful way to study its effects. Because it can be genetically transformed, poplar is amenable to this approach, unlike most woody plants. The genome of *P. trichocarpa* (black cottonwood) was recently published (Tuskan et al. 2006) making poplar a model system for molecular

biology and facilitating the identification and characterization of genes relevant to the synthesis of phenolics.

1.8.3 MYB 134 over expressing plants

The identification of the poplar transcription factor MYB 134 that regulates the entire condensed tannin biosynthetic pathway (Mellway et al. 2009) provides a unique opportunity for studying the impact of phenolics on phloem feeding insects. Overexpression of MYB 134 resulted in plants with 50 times higher levels of condensed tannins in leaves compared to untransformed wildtype controls (Mellway et al. 2009). The enhanced levels of tannins are the result of the upregulation of all enzymes of the flavonoid and condensed tannin pathway. An unexpected secondary effect of MYB 134 overexpression was a 2-3 fold reduction in phenolic glycosides, in particular tremulacin (Mellway et al. 2009). There were no visible or other major detectable chemical differences between transgenic and wildtype plants (Mellway et al. 2009). Since only a small number of studies have used transgenics to look at the effects of secondary metabolites on the fecundity of phloem feeding insects (Kaloshian and Walling 2005), these plants are a useful model for testing the effects of these changes in phenolics on insect herbivores.

1.8.4 Objectives, research questions and key findings

The objective of this project was to investigate if phenolic secondary metabolites affect phloem feeding insects, using transgenic poplar that produces high tannins and low phenolic glycosides and *Chaitophorus* aphids. The specific questions I aimed to answer are:

1. Do the shifts in tannin and phenolic glycoside profiles in the MYB 134 overexpressor plants affect the fitness of *Chaitophorus* aphids?

2. Do the shifts in tannin and phenolic glycoside profiles in the MYB 134 overexpressor plants affect the behaviour of *Chaitophorus* aphids?

3. Are phenolic compounds found in poplar phloem sap or in poplar feeding *Chaitophorus* aphids?

A no choice bioassay showed that *Chaitophorus* aphid fecundity is significantly higher on MYB 134 plants compared to on wildtypes. Conversely, when given a choice, aphids showed a significant preference for lower tannin leaf tissue. The phenolic glycosides salicin, salicortin and tremulacin were identified in poplar phloem sap exudates of both transgenic MYB 134 and wildtype plants. The presence of these phenolic glycosides in the extracts of aphids grown on both plant types provides the first direct evidence that these phenolic compounds are present in poplar phloem sap and that phenolic compounds can affect aphids.

Chapter 2. Effects of poplar phenolics on the fitness and behaviour of *Chaitophorus* aphids

2.1 Introduction

It is estimated that more than 20% of the annual net primary productivity of plants is removed by herbivores (Agrawal 2011). Insect herbivores alone consist of over one million taxonomically diverse species that use plants as a food source (Howe and Jander 2009). As sessile organisms that are unable to escape attack, plants have evolved defense strategies to protect against insects. One chemical strategy that plants use is the production of secondary metabolites, specialized compounds that are not required for growth or reproduction but have ecological functions.

Plants synthesize more than 200 000 secondary metabolites that are diverse in their structure and mode of action against insects (Mithöfer and Boland 2012). Secondary metabolites may be produced constitutively or induced by damage. They may act directly by exerting toxic, antinutritive or deterrent effects, or indirectly by attracting predators and parasitoids of their herbivores. Some major classes of secondary metabolites include alkaloids, terpenoids, glucosinolates, cyanogenic glycosides and phenolics.

Although the role of secondary metabolites in plant defense has long been studied, it is challenging for several reasons. First, plants produce a large number of secondary metabolites (Schoonhoven et al. 2005) and it is often difficult to study the effects of individual compounds; defense compounds also often act synergistically (Rasmann and Agrawal 2009). Second, the abundance of specific secondary metabolites is highly variable within species (Laitinen et al. 2000) as well as within an individual plant, and can be influenced by age (Donaldson et al. 2006), plant part (Kleiner et al. 2003), environmental factors (Lindroth and Nordheim 2001) and biotic interactions (Peters and Constabel 2002). Third, insect herbivores vary in their degree of host specificity and feeding style, both of which influence their exposure to and interactions with plant secondary metabolites (Kaloshian and Walling 2005; Ali and Agrawal 2012).

Despite these challenges, many studies have focused on the role of secondary metabolites in plant defense. Phenolics, in particular condensed tannins (herein referred to simply as tannins), have long been hypothesized to protect against insect herbivory since Feeny (1970) correlated seasonal patterns of caterpillar damage on *Quercus robur* (common oak) with foliar tannin concentration. It was hypothesized that the ability of tannins to bind protein leads to decreased nutrient availability for the insect. However, the basic pH of some insect guts (Fox and Macauley 1977) which prevents tannins from binding protein and the finding that protein digestibility was not affected for grasshoppers (Bernays et al. 1981), suggest alternative modes of action. Tannins may be toxic as prooxidants, which produce harmful oxygen radicals and quinones (Appel 1993) but evidence of this only exists for hydrolysable tannins, which are structurally different from condensed tannins (Barbehenn et al. 2001; Barbehenn et al. 2009). Tannins may also act as deterrents due to their astringent taste (Feeny 1970; Appel 1993). Experimental studies on the effects of tannins on insects have reported stimulatory, deterrent and no significant effects on feeding (Barbehenn and Constabel 2011). Thus, the effects of tannins on insect herbivores are variable and their mode of action is not clear.

Most research on tannins has focused on chewing insects, and little is known about how phloem feeders are affected by tannins (Barbehenn and Constabel 2011). Phloem feeding insects use specialized mouthparts called a stylet to feed on plant phloem sap within sieve tube elements (Walling 2008). Aphids are important phloem feeding agricultural pests. When feeding, the aphid stylet penetrates the leaf epidermis and passes intercellularly between mesophyll cells before reaching the sieve tube elements (Tjallingii and Esch 1993). The path the stylet takes is not always direct and nearly every cell along the stylet path is punctured. The cells tasted along this path determine what plant defenses aphids encounter (Walling 2008). Aphids counteract plant defenses by secreting sheath saliva along the stylet path; this acts to prevent slipping at the leaf surface, protect against defenses in the intercellular space (apoplast), and seal the damage site of the sieve tube element (Miles 1999). Given that phloem feeders have different feeding styles and strategies, they may be affected by phenolics differently than chewing insects.

Populus is a useful genus to study the effects of phenolics on insects because it contains a diverse range of phenolic compounds. These include high levels of tannins as well as the salicin based phenolic glycosides or "salicinoids" (from here on referred to simply as phenolic glycosides). There are approximately 20 phenolic glycosides in poplar and its relatives (Salicaceae) and the most commonly reported phenolic glycosides in *Populus* spp. (poplar) are salicin, salicortin, tremulacin and tremuloidin (Boeckler et al. 2011). Studies that take advantage of the large natural variation of phenolics among *P. tremuloides* genotypes have been instructive in understanding the role of phenolics in herbivore defense (Hwang and Lindroth 1997; Osier et al. 2000). However, direct proof of the role of these chemicals in defense is often lacking. Therefore, transgenic poplar provide a powerful approach to investigating the effect of a single or few phenolic compounds on insects.

The sequencing of the *P. trichocarpa* genome has facilitated the identification and characterization of genes relevant to phenolic biosynthesis. Recently, the poplar MYB 134 gene was shown to control the entire condensed tannin pathway and its overexpression in poplar saplings resulted in up to 50 times higher condensed tannin concentrations (Mellway et al. 2009). MYB 134 overexpression also resulted in 2-3 times lower levels of total phenolic glycosides, in particular tremulacin (Mellway et al. 2009). However, other branches of the flavonoid pathway (flavonols, anthocyanins) do not appear to be regulated by MYB 134. Also, transgenic plants did not show visible phenotypic differences or abnormalities (Mellway et al. 2009).

The objective of this study was to investigate the effect of changes in poplar phenolics due to MYB 134 overexpression on phloem feeding insects. To do this I used transgenic poplar that overexpress the MYB 134 gene and wildtype control plants in bioassays with *Chaitophorus* aphids. *Chaitophorus spp.* are specialists that feed exclusively within Salicaceae, with individual species feeding on either *Populus* or *Salix spp.* (Blackman and Eastop 1984). My results show that in poplar, phenolic compounds have significant effects on aphid fitness and behaviour. To demonstrate that aphids directly encounter and ingest phenolics when feeding on poplar, I also analyzed the extracts of whole aphids reared on both MYB 134-46 and wildtype control plants for the presence of phenolic compounds.

2.2 Materials and methods

2.2.1 Plant and aphid samples

Plants

P. tremula x *tremuloides* clone INRA 353-38 (wildtype) was originally provided by Steve Strauss (Oregon State University) and Richard Meilan (Purdue University) and transgenic *P. tremula* x *tremuloides* clone MYB 134-46 (MYB 134-46) was previously engineered by the Constabel lab (Mellway et al. 2009). Both lines were micropropagated in vitro on solid Woody Plant Media (Caisson Labs, USA) with 3% w/v sucrose, 0.1 mg/mL indolebutyric acid and 0.75% w/v Phytoagar (Sigma). *Populus trichocarpa* x *deltoides* clone H11-11 was originally provided by Gary Radamaker (University of Washington) and was macropropagated from greenwood cuttings. All plants were maintained in the University of Victoria's Bev Glover greenhouse under the growing conditions described in Major and Constabel (2006) with the exception of being watered daily without supplemental fertilizer.

Tannin assays were performed to confirm that tannin levels were elevated in MYB 134-46 plants as previously observed. Leaves were harvested from experimental plants, frozen in liquid nitrogen, lyophilized and stored at -80°C until analysis. Whole freeze dried leaves were ground to a fine powder with a mortar and pestle frozen with liquid nitrogen. For each leaf sample, 50mg of tissue was weighed and mixed with 10mL 100% methanol in a 15mL Falcon tube. Samples were shaken overnight for the extraction of tannins. Samples were then vortexed and centrifuged for 5 minutes at 4000 rpm and the supernatants were used directly for analysis. Tannin concentrations were determined with the colorimetric method of Porter et al. (1986) using purified *P. tremuloides* tannin as a standard obtained from Lynn Yip, University of Victoria.

Aphids

Chaitophorus sp. were collected in June 2010 from an ornamental hybrid *Populus sp*. growing outside the Engineering building on the University of Victoria campus in

Victoria, British Columbia. A 658 base pair fragment of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene using LepF (ATTCAACCAATCATAAAGATATTGG) and LepR (TAAACTTCTGGATGTCCAAAAAATCA) primers (Foottit et al. 2008) was amplified and sequenced showing that these aphids are members of an as yet unnamed *Chaitophorus* species that was introduced from Europe where it is normally found on *P. alba* (Dr. Robert Foottit, Agriculture and Agri-Food Canada Research Center, personal communication). A stock culture was established from a single founder colony on *P. trichocarpa* X *deltoides* stem cuttings. This stock culture was maintained in an incubator at 21°C with a 16:8 light:dark cycle. Preliminary experiments showed that these aphids readily fed on either of wildtype or MYB 134-46 experiment plants. Interestingly, other aphids that I collected from multiple locations in Victoria, BC, from local *P. tremuloides* and *P. trichocarpa,* and that appear to be closely related to *C. populifolii,* did not survive on any experimental plants. However, they did grow and reproduce on *P. trichocarpa* X *deltoides* in a growth chamber.

2.2.2 Effects of poplar secondary compounds on aphid fitness and behaviour

Aphid No Choice Experiment

Five, six week old wildtype and MYB 134-46 plants with approximately ten leaves were placed in separate fine mesh cages (length and width = 68.6cm, height = 96.5cm) in alternating order across two benches in the greenhouse. On 22 May 2012, seven, 18 day old apterous adult aphids were transferred onto leaf 5 of each experimental plant and allowed to reproduce. All aphids except for a single nymph were removed after 24 hours. These nymphs were allowed to mature into adults and their fecundity assessed as the number of offspring produced between 3 June 2012 and 26 June 2012. Offspring were counted and removed daily. The effect of plant genotype on aphid fecundity was assessed using a one-way analysis of variance (ANOVA) with genotype (wildtype or MYB 134-46) as the explanatory variable and aphid fecundity as the response variable.

Leaves 20 and 50 were frozen in liquid nitrogen, freeze dried and stored at -80°C until analysis for tannins. The average tannin level for each plant was calculated from

leaves 20 and 50 and the effect of plant genotype on average tannin level was assessed using the nonparametric Mann-Whitney U-test.

Aphid Choice Experiment

For each of five wildtype and five MYB 134-46 plants, 20 leaves were chosen and assigned to one of three age classes (young: leaves 2-8, medium: leaves 24-29, and large: 44-50). Leaf one refers to the second fully expanded leaf and so on. Plants were two and a half months old and had between 50 and 60 leaves. For each leaf, a leaf disc was cut from a wildtype plant and paired with a leaf disc of equivalent age and size cut from a randomly chosen MYB 134-46 plant. Leaf disc pairs were placed one centimeter apart abaxial side facing upwards in a petri dish on 1% agar. To control for differences between aphid individuals that could affect choice, genetically identical aphids reared on the same leaf and of the same age were used. One 13 day old apterous adult aphid was randomly chosen and placed in between the leaf discs. Its choice (wildtype, MYB 134-46 or agar) was recorded after 3, 24, 48 and 72 hours beginning on 27 April 2012. Petri dishes were stored in an incubator at 21°C with a 16:8 light:dark cycle. After all leaf discs had been cut, leaves 1, 23 and 51 from each plant were frozen in liquid nitrogen, freeze dried and stored at -80°C until analysis for tannins. The average plant tannin level for each plant was calculated from leaves 23 and 51 because leaf 1 had degraded during freeze drying for some plants. The effect of tannin level on aphid choice was assessed using a generalized linear mixed model with repeated measures (GLMM). The difference in tannin level between leaf disc pairs calculated as the average tannin level for the MYB 134-46 donor plant minus the average tannin level for the wildtype donor plant was considered a fixed factor and dish as a random factor.

2.2.3 Phenolic analysis of poplar leaves, phloem, and aphids

Phloem Sap Extraction

Phloem exudates from three wildtype and four MYB 134-46 plants were collected in June 2012 using a modified version of the method described in Dafoe et al. (2009). Plants were four months old and had several leaves removed but were otherwise healthy.

A 10cm stem section between leaves 35 and 45 was excised using a single sided razor blade and the leaves removed from the section. In order to prevent contamination from xylem sap, a 1mm section of bark was removed from the basal end of the stem using a double sided razor blade and the exposed wood was sealed with 90% lanolin/10% paraffin wax. The stem section was rinsed with distilled water and blotted dry before being placed in a 50mL tube with 600µL 50mM Tris (pH 8.0)/2mM ethylenediaminetetraacetic acid. Stems were left for 2 hours at room temperature for phloem exudation. To assess their purity, phloem exudates were analyzed for the carbohydrates sucrose, fructose and glucose. Sucrose is the predominant photoassimilate translocated in phloem sap and samples with sucrose making up at least 90% of the total sugar were considered to be of sufficient purity with minimal contamination from surrounding cells. Exudates were then evaporated, weighed and stored at -80°C until LC-MSMS analysis for phenolic compounds. The effect of plant genotype on phenolic glycoside levels was analyzed individually for each compound using a one-way ANOVA.

Carbohydrate Assays

Phloem exudates were assayed for sucrose, and glucose and fructose using a method adapted from Campbell et al. (1999). Sugars were enzymatically converted to glucose 6-phosphate. In a reaction catalyzed by glucose-6-phosphate dehydrogenase, glucose 6- phosphate was converted to gluconate 6-phosphate and nicotinamide adenine dinucleotide (NAD) converted to NADH. Sugars were quantified colorimetrically by measuring the absorbance of NADH at 340nm. For the sucrose assay, the method was modified by using invertase to convert sucrose to D-glucose and D-fructose. Hexokinase was then used to convert D-glucose to glucose 6-phosphate.

Analysis of phenolic compounds in aphids

In a preliminary experiment, three one month old wildtype and three MYB 134-46 plants were placed in mesh cages in alternating order across one bench in a greenhouse 8 June 2012. Sixty aphids of mixed ages were transferred to each plant and allowed to reproduce. Plants were rinsed with water regularly to prevent the accumulation of honeydew. Because phenolic glycosides are more abundant in young

developing leaves and tannin in mature leaves, aphids were collected from a young, medium and old aged leaf. On 16 July 2012, aphids feeding on leaves 1 and 20 were harvested using a fine paintbrush. Aphid samples and leaves 1 and 20 were frozen in liquid nitrogen, freeze dried and stored at -80°C until extraction for phenolics. Because aphids feeding on leaf 40 were not abundant enough, they and leaf 40 were collected 25 July 2012.

Freeze dried aphid samples were accurately weighed and homogenized twice for 45s at 5500 units in 1.4mL 80% methanol for extraction of phenolic compounds. Samples were sonicated for 15 minutes and centrifuged for 5 minutes at 10 000rpm. The supernatant was removed and this extraction was repeated three times. The pooled supernatant was dried, weighed and reconstituted in 80% methanol to make an extract concentration of 10mg/mL. In parallel, whole freeze dried leaves were ground to a powder using a mortar and pestle frozen with liquid nitrogen. For metabolite extraction, 50mg of tissue was weighed out and extracted using the same method as for the aphid samples except in 100% methanol.

In a separate experiment, aphids were collected a second time from each of two wildtype plants and four MYB 134-46 plants. This time, aphids were pooled from multiple mature leaves and one representative mature leaf per plant was collected. Aphids and leaves were extracted as above. All extracts were dried in a SpeedVac and sent to Jena, Germany for LS-MSMS analysis for phenolics.

LC-MSMS

The analysis of aphid extracts was performed by Dr. Michael Reichelt, Max Planck Institute for Chemical Ecology in Jena, Germany, using the following procedures. Chromatography was performed on an Agilent 1200 high performance liquid chromatography (HPLC) system (Agilent Technologies, Boeblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6mm, 1.8µm, Agilent, Waldbronn, Germany). Five microliters of extract reconstituted to 10mg dry weight (DW) extract/mL methanol were injected for each sample. Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-0.5min, 5%B; 0.5-4.5min, 5-90%B; 4.5-4.51min 90-100% B; 4.51-5min

100% B and 5.1-8.5min 5% B. The mobile phase flow rate was 0.8 ml/min. The column temperature was maintained at 25°C. An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ionspray voltage was maintained at -4500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 60psi, curtain gas at 25psi, heating gas at 60psi and collision gas at 7psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: *m/z* 288.8 →109.1 (collision energy (CE)-22 V; declustering potential (DP) -35 V) for catechin; *m/z* 284.9 →122.9 (CE -18V; DP -30V) for salicin; *m/z* 422.8 →123.1 (CE -30V; DP -55V) for salicortin; *m/z* 527.1 →123.1 (CE -34V; DP -55V) for tremulacin. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

2.3 Results

2.3.1 Effects of poplar secondary compounds on aphid fitness and behaviour

Aphid No Choice Experiment

To determine if MYB 134 overexpression affects aphid fitness, no choice tests were performed with MYB 134-46 plants with significantly higher average tannin levels (mean=82.56ug/mg DW) than wildtype plants (3.23 ug/mg DW) based on phytochemical analysis of leaves 20 and 50 (Mann Whitney U-Test, W=20, p=0.01587) (Figure 2.1). All nymphs were born 23 May 2012 and all reached reproductive maturity within 11-12 days, with the exception of one aphid on a wildtype plant that began reproducing at 15 days old. The daily total number of offspring produced by aphids on MYB 134-46 plants was consistently higher than that for aphids on wildtype plants over the course of the experiment (Figure 2.2). Fecundity was calculated as the total number of offspring produced between 3 June 2012 and 26, June 2012. At the end of these 23 days of

reproduction, aphid fecundity was significantly higher on MYB 134-46 plants than on wildtype plants (one-way ANOVA, $F=29.105_{1,7}$, $p=0.001014$) (Figure 2.3).

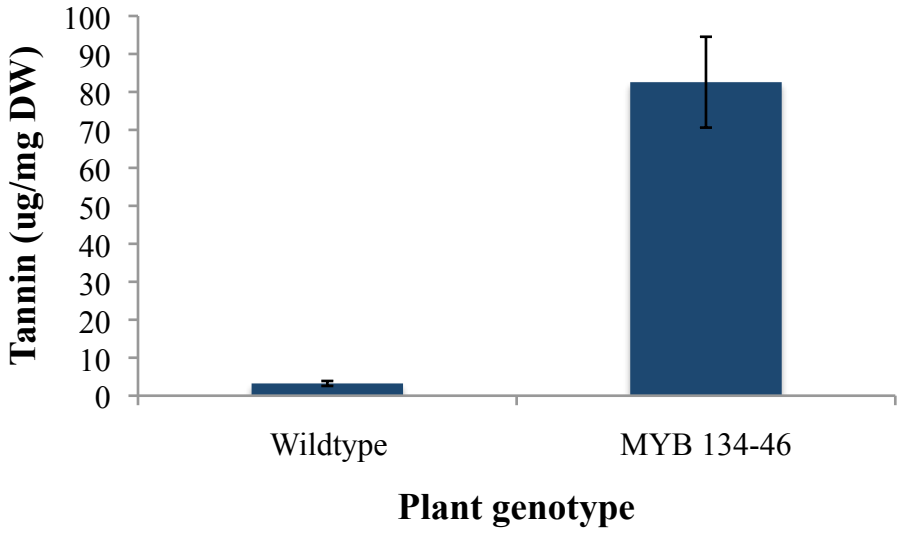


Figure 2.1 Experimental plant tannin levels for No Choice experiment. Wildtype plants have significantly lower tannin levels than MYB 134-46 plants (Mann Whitney U-Test, W=20, p=0.01587). Bars represent mean average tannin level for wildtype (n=5) and MYB 134-46 (n=4) plants based on leaves 20 and 50. Error bars represent +/- 1 standard deviation.

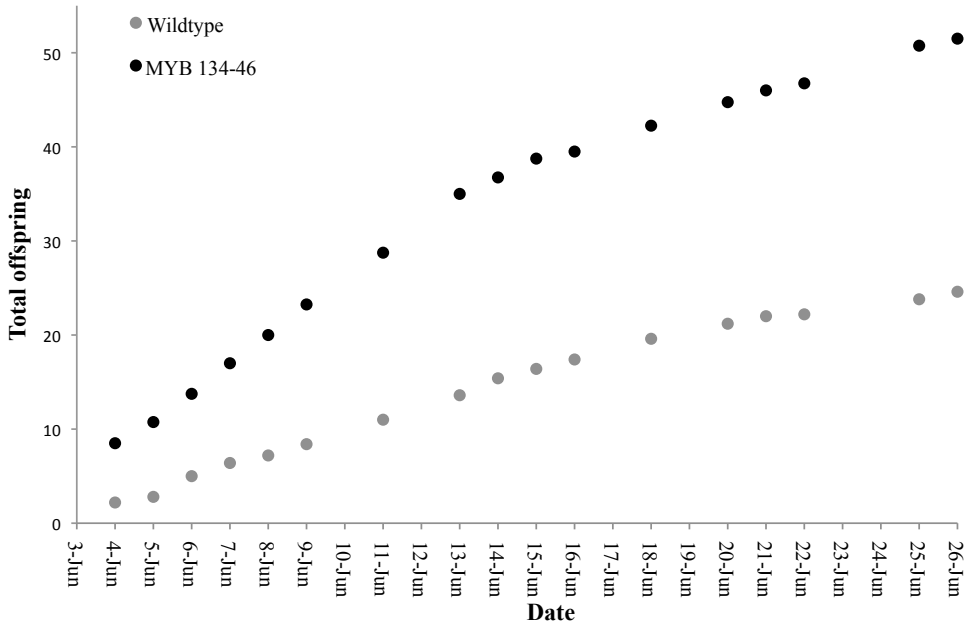


Figure 2.2 Daily average total offspring of aphids reared on either MYB 134 or wildtype plants (No Choice experiment). *Chaitophorus sp.* aphids growing on MYB 134-46 plants (n=4) have consistently higher daily average total offspring than aphids on wildtype (n=5) plants. Aphids were born 23 May 2012 on each experimental plant. Reproduction began 3 June 2012 and offspring were counted and removed daily until 26 June 2012.

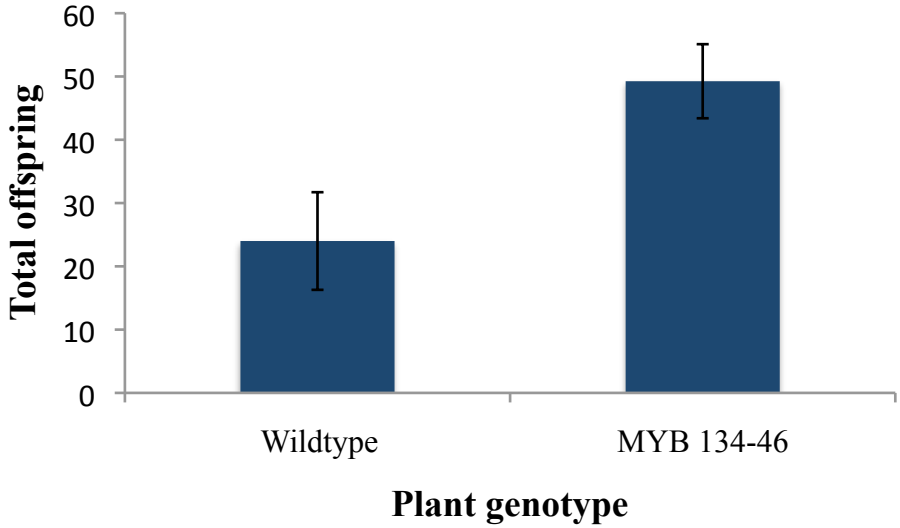
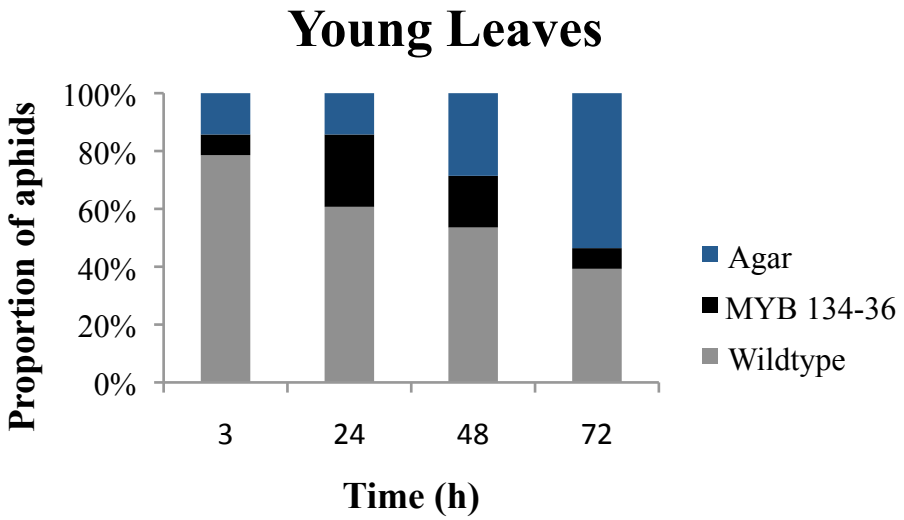


Figure 2.3 Total aphid fecundity during the first 23 days of reproduction on either MYB 134 or wildtype plants (No Choice experiment). *Chaitophorus sp.* fecundity is significantly lower on wildtype plants compared to on MYB 134-46 plants (one-way ANOVA, $F=29.105_{1,7}$, $p=0.001$). Fecundity was assessed as the total number of offspring produced between 3 June 2012 and 26 June 2012. Bars represent mean fecundity for aphids grown on wildtype (n=5) and MYB 134-46 (n=4) plants. Error bars represent +/- 1 standard deviation.

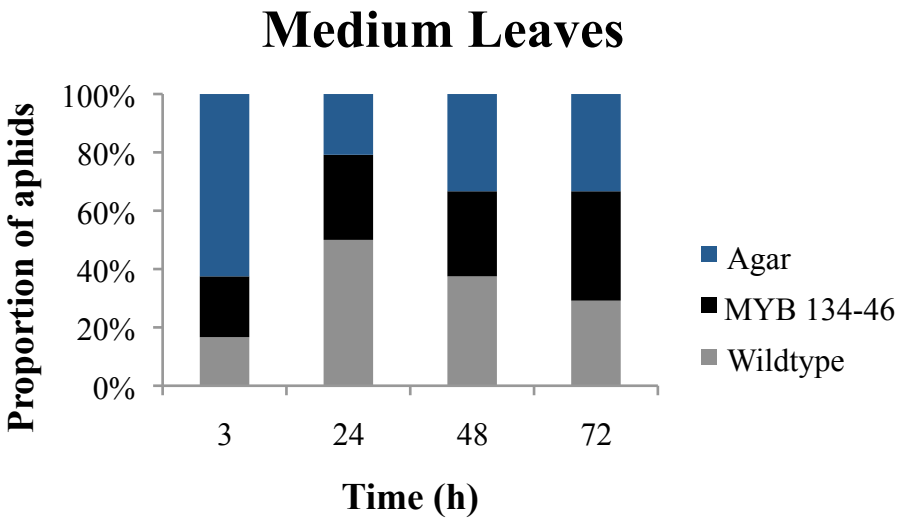
Aphid Choice Experiment

Because aphids performed better on MYB 134-46 plants, a choice test using excised leaf discs was performed to determine if aphids also prefer to feed on MYB 134-46 leaf tissue over wildtype tissue. MYB 134-46 plants had significantly higher tannin levels (mean=126.90 ug/mg DW), n=4) than wildtype plants (mean=32.34 ug/mg DW, n=5) (Mann-Whitney U-test, p=0.02). Since one MYB 134 plant had an unexpectedly low tannin level (3.37 ug/mg DW) all dishes with leaves from this plant were removed for visualizing the data in Figures 2.4a, 2.4b and 2.4c. All dishes were included in the analysis, which used the difference in tannin level between the pairs of wildtype and MYB 134-46 discs as the explanatory variable. Aphids showed an overall significant preference for lower tannin leaf tissue (GLMM, p=0.019). This preference appeared to be most prominent when aphids were given discs from old leaves, with 18 of 28 aphids

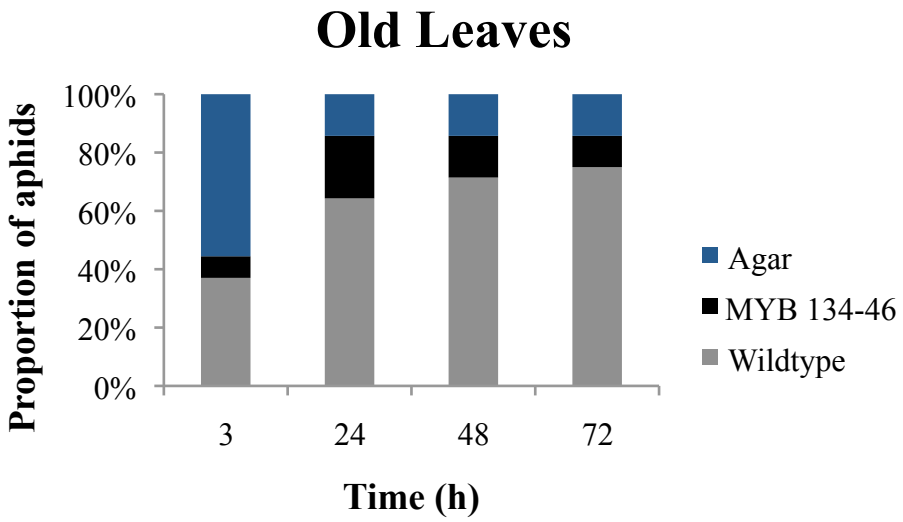
observed on the wildtype disc after 24 hours (and 6 aphids on the MYB 134-46 disc and 4 on the agar) and this number increasing over time (Figure 2.4c). Aphids also appeared more likely to settle on young and medium wildtype tissue after 24 hours, although over time they seemed increasingly dissatisfied with their food choices and moved onto the agar (Figure 2.4a, 2.4b).



a.



b.



c.

Figure 2.4 Proportion of aphids observed on wildtype discs, MYB 134-46 discs or agar during a 72 hour Choice experiment. Proportion of aphids observed on wildtype discs (gray), MYB 134-46 discs (black) or agar (blue) after 3, 24, 48 and 72 hours for (a) young, (b) medium and (c) old leaves.

2.3.2 Phenolic analysis of poplar leaves, phloem, and aphids

Phloem sap exudates

To determine if phenolic compounds are present in poplar phloem sap, which could explain the aphid fecundity and choice results observed in the bioassays, phloem exudates were collected from stem sections in EDTA and analyzed with LC-MSMS. The phenolic glycosides salicin, salicortin and tremulacin were identified in both wildtype and MYB 134-46 phloem exudates (Figure 2.5). MYB 134-36 phloem exudates contained significantly less salicortin (mean= 34.02 peak area counts/mg extract DW, n=4) than wildtype phloem exudates (mean= 7538.50 peak area counts area/mg extract DW, n=3) (one-way ANOVA, $F=7.0195_{1,5}$, $p=0.045$). MYB 134-46 phloem exudates also contained significantly less tremulacin (mean= 24.23 peak area counts/mg extract DW, n=4) than wildtype phloem exudates (mean= 3447.5 peak area counts/mg extract DW), n=3) (one-way ANOVA, $F=32.324_{1,5}$ $p=0.0023$) (Figure 2.5). These patterns are consistent with the reduced concentrations of phenolic glycosides in the leaves of MYB 134 overexpressor plants (Mellway et al. 2009). Although the purity of phloem exudates was assessed by

carbohydrate analysis, it is possible that damage to the surrounding cells during the excision could release phenolic glycosides into the phloem exudate using this approach. Therefore, the phloem sap origin of these phenolic glycosides in the phloem exudates cannot be proved directly. Because aphids feed primarily on phloem sap, the presence of phenolic glycosides in aphid bodies would be indicative of these compounds being in the phloem and could help explain the no choice and choice experiment results. Therefore, aphids were collected and analyzed for phytochemicals directly.

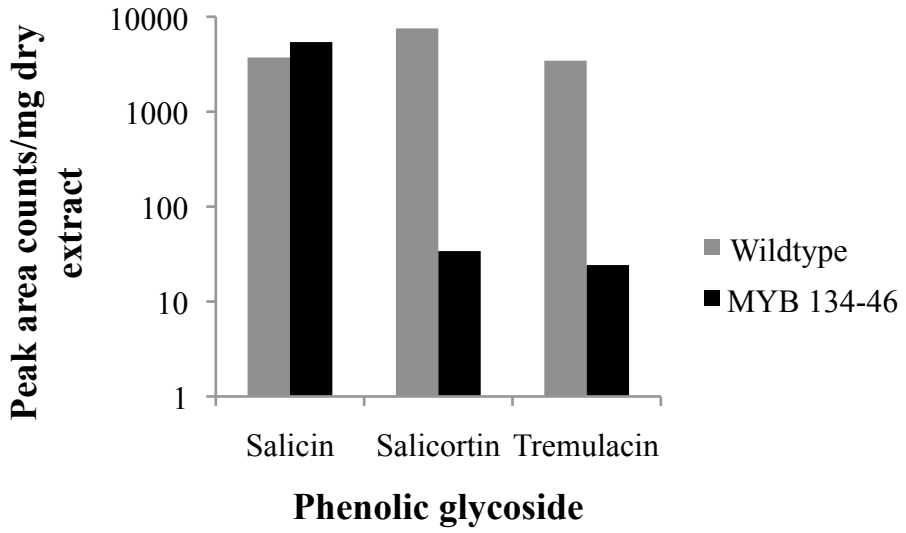


Figure 2.5 Phenolic glycoside levels in poplar phloem exudates collected by the EDTA method. Phloem exudates from both wildtype and MYB 134 plants were analyzed for salicin, salicortin and tremulacin content. MYB 134-46 phloem exudates contain significantly less salicortin (one-way ANOVA, $F=7.0195_{1,5}$, $p=0.045$) and tremulacin (one-way ANOVA, $F=32.324_{1,5}$ $p=0.0023$) compared to wildtype phloem exudates. Bars represent mean phenolic glycoside peak area counts/mg dry extract for wildtype (n=3) and MYB 134-46 (n=4) phloem exudates.

Aphid and leaf extracts

A preliminary experiment showed that phenolic metabolites were present in aphid extracts in very low but in highly variable concentrations as determined by LC-MSMS. This variation was not dependent on plant genotype (wildtype or MYB 134-46) or leaf age (leaf 1, 20 or 40) (Supplementary Table 2.1), and did not reflect the phenolic content of leaves (Supplementary Table 2.2). Therefore, aphids from multiple mature leaves were

pooled for extraction and analyzed in a subsequent experiment (Table 2.1). In parallel, one representative leaf from which the aphids were collected was also extracted and the major poplar phenolics assayed (Table 2.2).

The data indicate that aphids grown on both wildtype and MYB 134-46 plants contained catechin and the phenolic glycosides salicin, salicortin and tremulacin. Compared to leaf extracts, levels of these compounds detected in aphid extracts were lower, and for some compounds, near the detection limit. Catechin appears to be found at higher levels in aphids grown on MYB 134-46 plants compared to aphids grown on wildtype plants. This may reflect the higher levels of catechin in the leaves of MYB 134-46 plants (Table 2.2). Salicin was the most consistently detectable phenolic glycoside. Salicortin was detected in some samples, but just at the detection limit. This is consistent with the apparent absence of salicortin in the preliminary experiment, when aphid extracts were analyzed by ion trap, which is less sensitive (Supplementary Table 2.1). As salicortin is very abundant in leaves (Table 2.2), but barely detectable in aphids (Table 2.1), it may be preferentially or rapidly degraded or modified during digestion. Peaks of unidentified compounds with similar sizes to salicortin were detected in aphid extracts (not shown). It is possible that these could be salicortin derivatives. Despite the differential stability or potential degradation of phenolic glycosides in aphids, they are clearly present in the bodies of aphids. This confirms that they are ingested during feeding and supports their presence in poplar phloem sap.

Table 2.1 Phenolic analysis of aphids grown on wildtype and MYB 134-46 plants. Phenolic analysis of aphids grown on leaves pooled from wildtype and MYB 134-46 plants. Aphids were grown on two wildtype plants and four MYB 134-46 plants. Aphids from several mature leaves were pooled, frozen in liquid nitrogen, lyophilized and extracted in 80% methanol. Aphid extracts were analyzed for phenolics using Triple Quad LC-MSMS. Peak area counts for each compound are normalized to aphid extract dry weight (peak area counts/mg dry extract).

Genotype	Plant	Catechin	Salicin	Salicortin	Tremulacin
Wildtype	1	772	15200	903	1400
	2	0	2266	0	141
MYB134-46	1	2185	1944	230	241
	2	112	1664	0	163
	3	1206	5388	476	759
	4	89	417	0	30

Table 2.2 Phenolic analysis of leaves used to rear aphids. Leaf extracts contain catechin, salicin, salicortin and tremulacin at higher levels than aphids. One representative mature leaf was collected from each of two wildtype and four MYB 134-46 plants. Leaves were frozen in liquid nitrogen, lyophilized, extracted in 100% methanol and analyzed for phenolics using Triple Quad LC-MSMS. Peak area counts are normalized to leaf extract dry weight (peak area counts/mg dry extract).

Genotype	Plant	Catechin	Salicin	Salicortin	Tremulacin
Wildtype	1	3270	186772	424868	159788
	2	780	176606	307798	122018
MYB134-46	1	74510	106863	244608	90196
	2	55066	120705	278855	129515
	3	55385	140000	291282	145128
	4	60000	114286	232381	103333

2.4 Discussion

To investigate how phenolics affect phloem feeding insects, aphids were used in bioassays with poplar plants overexpressing the transcription factor MYB 134 that increases tannin and decreases phenolic glycoside levels in leaves (Mellway et al. 2009). These changes in phenolics had a significant effect on aphid fitness and behaviour. In a no choice experiment in which aphids were reared on whole wildtype and MYB 134 plants, aphid fecundity was significantly higher on MYB 134-46 plants than on wildtype plants. When presented with a feeding choice of wildtype or MYB 134-46 leaf discs, aphids showed a significant preference for lower tannin leaf tissue and this preference was more pronounced with older leaves. To investigate if these effects could be caused by poplar secondary metabolites, I looked for differences in phenolics in phloem exudates and in extracts of aphids grown on both plant types. Extracts prepared from whole aphids contained catechin and the phenolic glycosides salicin, salicortin and tremulacin. This provides evidence that these phenolics are present in poplar phloem, confirms that they are ingested during aphid feeding and suggests that they can influence aphid performance and behaviour on poplar.

2.4.1 Aphid fecundity is higher on MYB 134-46 plants than controls

In the no choice experiment, aphid fecundity was significantly greater on MYB 134-46 plants than wildtype plants. This difference in fitness may be due to physical or chemical differences between plant types. MYB 134 overexpression does not lead to visible phenotypic differences (Mellway et al. 2009), so it is unlikely that aphids are responding to physical properties that may enhance feeding. Rather, aphids could be responding to either the higher levels of tannins (or related compounds) or lower levels of phenolic glycosides, which are the main characteristics of MYB 134 transgenic plants.

It is unlikely that tannins increase aphid fecundity for two reasons. First, aphid extracts contained only trace levels of catechin, a condensed tannin precursor. The analysis of poplar phloem exudates also showed only low levels of catechin (Supplementary Table 2.3) making it unlikely that larger polymeric tannins are in the

phloem. However, it is also possible that catechin is modified during aphid digestion, perhaps by oxidative enzymes. Aphid oxidation of catechin by catechol oxidase and peroxidase secreted by the gut has been shown in *Macrosiphum rosae* (rose aphid) (Peng and Miles 1991). Second, a previous study on *Aphis craccivora* feeding on *Arachis hypogaea* (groundnut) showed a negative correlation between tannin levels and aphid fecundity (Grayer et al. 1992). Therefore, tannins are less likely to be driving the differences in aphid fecundity on MYB 134 and wildtype plants.

Instead, the hypothesis that higher levels of phenolic glycosides in wildtype plants decrease aphid fitness appears more likely. Previous work has shown that phenolic glycosides reduce insect fitness, although these studies have been limited to chewing insects (Boeckler et al. 2011). In work done on *M. disstria* and *L. dispar* larvae feeding on natural isolates of *P. tremuloides*, growth rates and developmental times were negatively and positively correlated with phenolic glycosides respectively (Hwang and Lindroth 1997; Osier et al. 2000). Artificial diets have also been useful in separating the effects of phenolic glycosides from other factors and demonstrating their relative toxicity, with salicin, salicortin and tremulacin all shown to decrease growth rates and increase developmental times for caterpillars and/or beetles (Lindroth and Peterson 1988; Kelly and Curry 1991; Hemming and Lindroth 1995). Salicin appears to be the least potent having non significant (Lindroth and Peterson 1988; Lindroth et al. 1988a) or less potent effects than salicortin (Kelly and Curry 1991). Synergistic effects between salicortin and tremulacin have also been described (Lindroth et al. 1988b). No studies have tested for the effects of phenolic glycosides on phloem feeders. Work on the effects of phenolic glycosides on phloem feeders is limited to two studies on aphid choice, which I will discuss in a later section.

Future work will be needed to test whether phenolic glycosides decrease *Chaitophorus* fitness, and to identify which compounds have the greatest effect. The hypothesis that phenolic glycosides are the cause for the differential fecundity is supported by the identification of salicin, salicortin and tremulacin in our phloem exudates. As mentioned previously, although I cannot rule out their presence in exudates by contamination from other cell types, their presence in aphid extracts confirms that they were ingested during feeding. We detected very low and inconsistent levels of salicortin

in our aphid extracts suggesting that it is degraded or modified. I propose that aphid performance is better on MYB 134-46 plants because their phloem contains significantly lower levels of tremulacin, which has potent effects against chewing insects (Hemming and Lindroth 1995). Other secondary metabolites present in phloem sap including glucosinolates have been shown to affect aphids (Kim et al. 2008). It will be important to determine what compounds the species of *Chaitophorus* used in this study encounters in its natural diet; interestingly, tremulacin has not been reported in *P. alba* (Boeckler et al. 2011).

2.4.2 Aphids prefer lower tannin leaf tissue

Although aphids perform better on MYB 134-46 plants, in the choice experiment they preferred lower tannin tissue. To understand this apparent contradiction in behaviour, it is important to consider how they feed. Aphids use modified mouthparts collectively called a stylet to feed on phloem sap located within sieve tube elements. Before inserting into a sieve tube element, the aphid stylet passes through the cuticle and between epidermal and mesophyll cells of the plant leaf. Detailed studies of aphid feeding using the electrical penetration graph technique show that the aphid stylet probes into cells along its path to the sieve tube elements (Martin et al. 1997; Tjallingii and Esch 1993). During this probing, cell sap is taken up into the stylet food canal (Martin et al. 1997) and likely into the epipharyngeal gustatory organ (Powell et al. 2006). This probing is likely limited to the cytoplasm of cells. This "tasting" of cells along the stylet path to a sieve tube element prior to ingestion of phloem sap could influence aphid acceptance of host plant tissue (Powell et al. 2006). A study looking at *Aphis fabae* (black bean aphid) reproduction showed that parturition, a sign of host acceptance, was related to cues from peripheral cells before contact with phloem sap (Tosh et al. 2002). The cytoplasm is the site of synthesis for many secondary metabolites including the tannin subunit catechin (Xie and Dixon 2005). In the choice test, aphids could be responding to the secondary metabolites in poplar mesophyll cells.

Chaitophorus aphids used in the choice experiment may thus be choosing lower tannin leaf tissue because they are deterred by higher levels of catechin in the cytoplasm of MYB overexpressor mesophyll cells during probing. Levels in phloem are likely too

low to act as a deterrent. Staining of leaf sections with dimethylaminocinnamaldehyde (DMACA), which reacts with flavan-3-ols and tannins to form a blue chromophore, showed that these are more abundant in the palisade and spongy mesophyll cells of MYB overexpressors (Mellway et al. 2009). When added to artificial diets at concentrations higher than 0.3mg/mL, catechin deterred *Macrosiphum rosae* (rose aphid) (Peng and Miles 1988). Although they have a different feeding style than aphids, it is noteworthy that tannins have deterrent effects on generalist and grass feeding grasshoppers (Bernays et al. 1981) and southern armyworm larvae (Manuwoto and Scriber 1986).

It is also possible that aphids are responding to phenolic glycosides in the phloem sap. Few studies have examined the effects of phenolic glycosides on phloem feeding insects; only two have examined how leaf chemical properties (nutrients, phenolics) affect aphid choice. *Populus angustifolia* leaves that had galls of the specialist galling aphid *Pemphigus betae* had more total phenolics than leaves without galls (Zucker 1982). Aphids also colonized leaves with lower levels of total phenolics, suggesting that phenolics may play a role in aphid leaf preference. However, this study did not exclude other factors such as nutritional value and did not distinguish between phenolic glycosides and other phenolics, such as catechin. In a more recent study, Gould et al. (2007) showed that salicin content was highest in phloem exudates of young *Populus deltoides* leaves and that *Chaitophorus populicola* showed a preference for feeding on young leaves, suggesting that salicin does not act as a feeding deterrent for these poplar aphids.

2.4.3 The presence of phenolic glycosides in phloem exudates and aphids

Our data suggest that phloem exudates contained salicin, salicortin and tremulacin, with the latter two being significantly more abundant in wildtype phloem exudates. If confirmed, it would be the first time salicortin and tremulacin have been reported in poplar phloem. Salicin was previously measured in the phloem sap exudates of *P. deltoides* (Gould et al. 2007). Aphid extracts also contained very low and variable levels of catechin, salicin, salicortin and tremulacin. The variation in these phenolics in aphid extracts did not appear to reflect the phenolic levels in the leaves on which they

were reared. The variation of these compounds in aphids may be driven by aphid feeding behaviour or metabolism.

Aphids may be consuming more or less phenolic containing phloem sap. Phenolic glycosides have been shown to affect the feeding behaviour of chewing insects. When painted on *P. tremuloides* and *Phaseolus lunatus* (lima bean) leaves, tremulacin decreased *Papilio glaucus glaucus* (swallowtail butterfly) and *S. eridania* larvae consumption (Lindroth and Peterson 1988; Lindroth et al. 1988b). In contrast, *M. disstria* consumed more when fed *P. tremuloides* leaves painted with a mixture of tremulacin and salicortin (Hemming and Lindroth 2000). However, this effect was reversed when larvae were also fed protein (Hemming and Lindroth 2000).

An alternative explanation for the differences in phenolic glycoside levels between aphid extracts is that aphids may be modifying them post ingestion, as in the case of *Operophtera burmata* (winter moth), which has been shown to break down salicortin and salicin after feeding on *Salix myrsinifolia* (willow) (Ruuhola et al. 2001). It is interesting to note that salicortin is abundant in poplar leaves and was found in phloem exudates and aphid extracts at very low levels. This suggests that it may also be degraded by *Chaitophorus*. Aphid extracts had unidentified peaks with fragment patterns similar to salicortin (not shown). It is possible that these are breakdown products or conjugates of salicortin, although further investigation is necessary to confirm this. Although phenolic glycosides can be degraded by certain insect herbivores, how they are metabolized is still unknown. Two enzymes are thought to be involved: beta glycosidases for salicin, and esterases for more complex phenolic glycosides (Boeckler et al. 2011); both enzymes use phenolic glycosides as substrates in vitro (Julkunen-Tiitto and Meier 1992).

Finally, it will be interesting to determine if *Chaitophorus* aphids use phenolic glycosides to defend against natural enemies, as has been shown in the specialist beetles *Phratora vitellineae* and *Chrysomela tremulae* that feed on willow and poplar, respectively. These beetles use salicin found in leaves to manufacture salicylaldehyde, a compound that protects them against predators (Pasteels et al. 1983). It is not known whether aphids can sequester phenolic glycosides but *B. brassicae* is able to sequester glucosinolates in its hemolymph and make its own myrosinase enzyme that is stored separately (Kazana et al. 2007).

2.5 Summary

There are very few studies that show that phloem sap contains phenolic compounds (Gould et al. 2007) and that test if these affect phloem feeding insects (Grayer et al. 1992). This is in contrast to other secondary metabolites such as glucosinolates (Chen et al. 2001; Kim et al. 2008), alkaloids (Wink and Witte 1984; Wink et al. 1992; Lee et al. 2007) and cyanogenic glycosides (Calatayud et al. 2004; Jorgensen et al. 2005).

My work shows that changes in phenolics due to MYB 134 overexpression affect *Chaitophorus* fitness and behaviour. Although aphid fecundity is higher on MYB 134 plants, they prefer lower tannin leaf tissue. I hypothesize that aphids are responding to lower levels of tremulacin in MYB 134-46 phloem and higher levels of catechin in the mesophyll cells surrounding the sieve tube elements in wildtype plants. This is the first study to report salicortin and tremulacin in poplar phloem exudates. My finding that salicin, salicortin and tremulacin are present in aphid extracts provides evidence for these compounds being in poplar phloem sap. This is also the first study to identify these phenolic glycosides in a phloem feeding insect. Therefore my contribution is expanding the list of secondary metabolites in phloem sap to include phenolics and showing that these can affect aphids feeding on poplar.

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Appendix A. Supplementary Tables

Supplementary Table 2.1 Preliminary analysis of phenolic compounds in aphids. Aphids were grown on three wildtype and three MYB 134-46 plants and aphids feeding on leaves 1, 20 and 40 were frozen in liquid nitrogen, lyophilized and extracted in 80% methanol. Extracts were analyzed for phenolics by Ion Trap LC-MSMS. Peak area counts are normalized to aphid extract dry weight (peak area counts/mg dry extract).

Genotype	Plant	Leaf	Catechin	Salicin	Salicortin	Tremulacin
Wildtype	1	1	0	1071593	0	3371396
	1	20	0	223335	0	0
	1	40	0	278737	0	0
	2	1	0	33704	0	0
	2	20	0	0	0	0
	2	40	0	0	0	0
	3	1	0	366882	0	0
	3	20	0	339138	0	0
	3	40	0	0	0	0
MYB134-46	1	1	0	0	0	0
	1	20	0	274242	0	0
	1	40	0	0	0	0
	2	1	0	347971	0	0
	2	20	290459	1318309	0	2903208
	2	40	0	1025727	0	5734400
	3	1	562367	1643972	0	4375940
	3	20	0	0	0	0
	3	40	0	361174	0	0

Supplementary Table 2.2 Preliminary analysis of phenolic compounds from leaves used to rear aphids. Leaves 1, 20 and 40 were harvested after all aphids were removed. Leaves were frozen in liquid nitrogen, lyophilized and extracted in 100% methanol. Extracts were analyzed for phenolics by Ion Trap LC-MSMS. Peak area counts are normalized to leaf extract dry weight (peak area counts/mg dry extract).

Genotype	Plant	Leaf	Catechin	Salicin	Salicortin	Tremulacin
Wildtype	1	1	0	3437406	79112965	84493068
	2		0	2601851	105848031	101151595
	3		0	4563196	87809025	105362745
	1	20	27406	177012	6879719	5148068
	2		219088	184078	5945819	5117241
	3		33016	129790	4102417	3658720
	1	40	15746	253913	4829087	3471186
	2		15732	219519	4438723	3563246
	3		346544	2373561	44682180	34308476
MYB134-46	1	1	711782	3525518	114855986	117598665
	2		975027	2948899	80112238	83627353
	3		1706132	2767414	81711908	95315401
	1	20	217704	142838	3656449	2936599
	2		284301	135337	3267306	2520882
	3		251373	131818	3358142	3195168
	1	40	2357916	185411	2897489	2419673
	2		229365	171894	2943792	2385710
	3		210738	152541	2463055	2108879

Supplementary Table 2.3 Analysis of phloem exudates for phenolic compounds. Phloem exudates contain salicin, salicortin tremulacin and very low levels of catechin. Phloem exudates were collected from stem sections in EDTA. All extracts were assessed for purity and sucrose made up at least 90% of the total sugars. Exudates were analyzed for phenolics using Triple Quad LC-MSMS. Peak area counts are normalized to phloem exudate dry weight (peak area counts/mg dry extract).

Genotype	Plant	Catechin	Salicin	Salicortin	Tremulacin
Wildtype	1	1703	4912	5118	3618
	2	630	3500	3273	2125
	3	685	2750	14225	4600
MYB 134-46	1	2337	12405	32	55
	2	702	2744	55	6
	3	155	3633	14	14
	4	138	2868	36	22