Searching for resistance to willow sawfly: the importance of chemicals

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Introduction

Many secondary plant compounds play a role in deterring herbivores. Some secondary compounds inhibit specific steps in mitochondrial respiration, including, for example, HCN, which is released from cyanogenic compounds that are present in a wide range of species, which blocks cytochrome oxidase. Other secondary compounds are much less specific; for example, tannins, which precipitate proteins and thus interfere with food digestion. Toxic phenolic glycosides as found in *Populus* and *Salix* species deter herbivores, are another example of these less specific secondary compounds. Salicin, one of these phenolic glycosides, is hydrolysed and oxidised after ingestion, producing salicylic acid, which uncouples oxidative phosphorylation in mitochondrial preparations. The structure of phenolic glycosides resembles that of many allelopathic compounds, which suggests that the selective driving force for their formation may well have been their role in deterring herbivores or pathogens. Both the total phenolic glycoside concentration in the leaves and the spectrum of these compounds varies among *Salix* species. Phenolic glycoside concentration in the leaves has been correlated with the selective feeding behaviour of herbivorous beetles (Tahvanainen et al, 1985; Orians et al, 1997; Ikonen et al, 2002) and southern armyworm larvae (Lindroth and Petersen, 1988). Generalist feeders show a preference for leaves with low phenolic glycosides, whereas some specialist feeders which restrict themselves to one or two species show a much greater adaptability to leaves with high phenol glycoside levels. Physical damage of leaves often enhances the transcription of genes encoding polyphenol oxidase (e.g., in *Populus* species) (Constabel et al. 2000). It also leads to the formation of tannins, and induces the production of protease inhibitors, especially when it is due top insect attack (Koiwa et al. 1997). These plant responses reduce the quality of both the attacked and other leaves on the same plant as a food source. This response sometimes occurs within minutes to hours (short-term induction), as a result of reactions among precursors already in the leaf. For example, chewing of quaking aspen (*Populus tremuloides*) leaves causes enzymatic hydrolysis of two phenolic glycosides (salicortin to salicin, and tremulacin to tremuloidin) with the release of 6-HCH, which then becomes converted to phenol or catechol (potent toxins) in the gut of the insect (Clausen et al. 1989). As a result, insects cannot feed continuously on a few leaves; rather, they must constantly move among leaves, which makes them more vulnerable to predators.

There are also long-term induced defenses produced by the next cohort of leaves after severe insect outbreaks. These serve to protect plants against catastrophic herbivory by insects with large population outbreaks. Long-term induction is typically associated with increases in phenolics, less leaf nitrogen and often smaller leaves

Selection for *Salix* species high in phenolic glycosides then, as a strategy for reducing the impact of the willow sawfly larva, a generalist feeder, is supported by the scientific literature. Identifying the levels of both specific and total phenolic glycosides contained within particular species, and making associations with willow sawfly behaviour and physiology is an important first step in this process. Likewise, searching for evidence of short-term and long-tern induced responses by willow plants to simulated insect feeding through a change in specific or general phenol glycoside concentrations will support our understanding about the resourcefulness of the various *Salix* species to counteract willow sawfly larval attack.

A: Sawfly development

Methods

Measurement of sawfly development on selected willows is continuing during 2002-03 in order to discover links between development (or inhibition of development) and secondary leaf chemicals in the different willow species or clones. *N. oligospilus* were reared on willows for a complete generation in the laboratory.

The host plants were grown from dormant cuttings at HortResearch, Mt Albert Research Centre. Leaves of standardised maturity and external appearance were selected from each host plant, and fed to a total of 20-40 willow sawfly larvae under a standardised environmental regime (nominally 23^{o}C and a 16h photoperiod). The experiment was repeated 3 times (during November, January and March) to mimic the impact of seasonal changes in host plant on sawfly development. Larval developmental times (from newly hatched larvae to adult emergence) and resulting adult fecundity when reared on each species were measured and compared. To measure fecundity, each female was dissected, her ovaries removed, and the number of ovarioles, immature and mature eggs counted.

A sample of leaves from each host plant during November-April, omitting February, was sent to Dr. D. Rowan at Palmerston North for phenol glycoside analysis. The intention was to compare larval development and adult fecundity with the type and quantity of phenolic glycosides in the leaves.

B: Phenolglycosides

The phenol glycoside analysis experiment focussed on

(a) the seasonal variability in phenolglycoside (PGS) concentrations, and
(b) the effect of varying severity of defoliation on the PGS concentrations following defoliation

Leaf samples were collected monthly from both defoliated and undefoliated plants. Treatments and sampling methods are detailed below.

Sampling Methods:

1 CONTROL PLANTS

Three leaves from leaf range 6-12 on a branch were sampled monthly from December – April for selected species from Palmerston North and Mt Albert (omitted sampling in February). Three trees were sampled at Palmerston North and one tree at Mt Albert.
Species being treated were ‘Kawa’, and the willows PN 227, PN 229, PN 249, PN 236, PN 386, PN 698, PN 741, PN 745 and PN 751 (see Table 1)

2 LIGHT DEFOLIATION

Monthly Treatment: For leaves 6-12 half the leaf is removed with scissors producing a defoliation of a third for leaves 6-12 from each branch.
Monthly sampling: 3 half-leaves taken the next day from defoliated trees.
Species being treated are ‘Kawa’, PN 741, PN 386, PN 249, PN 227 and PN 751

3 HEAVY DEFOLIATION

Monthly treatment: Two treatments were applied on Day 0 (a) for a complete branch whole leaves are removed with scissors leaving the central vein only (b) for a complete branch half leaves were removed with scissors
Monthly sampling: (a) 3 whole leaves were sampled from an untreated branch on Day 1, Day 3 and Day 7 following treatment (b) 3 half leaves were sampled from a treated branch on Day 1, Day 2 and Day 7.
Species being treated is PN 745.

Sampled leaves were picked, stored in Minigrip bags with freezer packs in a chillibin and transferred to a freezer at -10° C until they were processed for chemical extraction.

Chemical Extraction:

The stored leaves were immersed in liquid nitrogen, ground, stalks removed and then weighed. The weighed leaf material was immersed in 3-10 ml methanol containing arbutin standard (arbutin concentration = 2.0062 mg / ml, diluted 1:9 in MeOH) and further ground using an Ultraturrax grinder to dissolve the phenolglycosides. Three mls were pipetted off from the resulting mix and diluted to 80% MeOH. The chlorophylls were removed by passing the diluted liquid through a Varian bond Elut C18 filter. The final filtrate was analysed for phenolglycosides using high pressure liquid chromatography (HPLC).

Results:

Species variation in selected phenolglycosides(PGS)
Among the species we are sampling the analysis of key PGS present shows some marked differences (Table 2). In particular, there is considerable variation in salicin (nil – very high) and salicortin. Low levels of salicin are accompanied by low levels of tremulacin in *Salix schwerinii, S. medemii* and *S. exigua*. Conversely in the other species, excepting Kawa, levels of salicin and tremulacin are both high. Both of these chemicals are considered to be active in *Salix* defence against herbivory, either as precursor or end-product. Salicin is a building block for more toxic PGS molecules such as salicortin as well as a hydrolysis product, and tremulacin is readily converted to the more toxic tremuloidin (Clausen, 1989).

Geographical variation in selected phenolglycosides
PGS levels do not differ greatly between the two geographical locations, any difference being within the same order of magnitude (Table 3). Differences are more closely linked with genetics than geographical location, confirming our 2002 study.

Seasonal variation in selected phenolglycosides
Levels of salicin, tremulacin, salicortin and total PGS varied over the five months in which sampling occurred (Figure 1 a-c, Table 4). The levels appeared to peak during summer and then fall again, but there was not a consistent pattern for all chemicals or all species.

Phenolglycosides and leaf defoliation treatments

Light defoliation
Changes in the levels of salicin, tremulacin and total phenolglycosides were analysed for the six species tested (Table 5). Neither salicin or tremulacin was present in *S. schwerinii.* Tremulacin was present in samples of 'Kawa' only in December andJanuary, and in samples of *S. matsudana* only in April and March. Any changes in the concentration of these key phenolglycosides in response to the light defoliation are either insignificant or developed

outside of the sampling time of 24 hours. Short term responses triggered by insect feeding are reported to happen with in a few hours (see introduction) but may require repeated "insect' stimulation to maintain.

Heavy defoliation

Following heavy defoliation leaves were sampled after 1, 3 and 7 days in order to detect any long term response as described in the introduction. Heavy defoliation was carried out with one species, *S. lasiandra.* There was a similar pattern in changes in salicin and total phenolglycoside levels in the leaves following half leaf defoliation (figures 2 and 3) but not following whole branch defoliation. Analysis of the data using ANOVA is summarised in Table 6. Only the changes in salicin were significant at $p < 0.05$. The salicin response appears to follow the pattern of a long term response to insect feeding. Whether the salicin rise is due to increased production or is a result of enzymatic oxidation of salicortin or possibly other phenolglycosides is not entirely clear. Evidence from changing levels of , in particular, salicortin (Figure 4) favours the suggestion of increased salicin production, which is then transformed into other defensive phenolglycosides. It is reasonable to assume that not all salicin will be converted to salicortin. However, the fall in salicin levels on Day 7 occur simultaneously with a rise in salicortin levels in the months December – February.

Discussion:

There is considerable variability in the concentration of the various identifiable phenolglycosides between willow species, ranging from absent to very high levels. These differences in concentrations need to be correlated with larval maturation times and female fecundity.

Seasonal changes in phenolglycosides may be responding to available photosynthate, which peaks over the summer months when leaf area reaches a maximum and leaf tissue production is reducing. Phenolglycoside production is costly to the plant, particularly in terms of N. This emphasises the serious impact of early season defoliation by the sawfly larvae on the ability of the tree to provide photosynthate for the production of defence chemicals as well as new tissue.

Analysis of HLD and WBD showed that the plant response is quite different for these two treatments. With HLD the plant retains damaged leaves which can respond by i. increased production and conversion of chemical defenses, and ii. by production of a translocated signal that could stimulate production in undamaged leaves, whereas with WBD there are no damaged leaves remaining on the plant so neither of these responses are likely to happen (Constabel et al, 2000).

This series of experiments has produced some encouraging evidence aligning the levels of, in particular, salicin and salicortin with a long term response to physical leaf damage such as would be produced by willow sawfly herbivory. These two compounds,as well as tremulacin and tremuloidin (for which we were unable to identify HPLC peaks) have been reported as being active in plant defenses against herbivory (Clausen et al, 1989; Ikonen et al, 2002; Lindroth and Petersen, 1988; Orians et al, 2003). These studies have followed up this approach with the feeding of specific phenolglycosides to larvae either painted onto leaves or soaked in discs of filter paper to link retardation in insect development with a specific phenolglycoside(s). This would be a sensible next step in conjunction with the breeding of clones showing some resistance.

References:

Clausen TP, Reichardt PB, Bryant JP, Werner RA, Post K, and Frisby K. 1989. A chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. J. Chem. Ecol. 15: 2335-2346.

Constabel CP, Yip L, Patton JJ and Christopher Me. 2000. Polyphenol oxides from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiol. 124: 285-295.

Ikonen A, Tahvanainen J, and Roininen H. 2002. Phenolic secondary compounds as determinants of the host plant preferences of the leaf beetle, *Agelastica alni*. Chemoecology 12: 125-131.
Koiwa H, Bressan RA, and Hasegawa PM. 1997. Regulation of protease inhibitors and plant defense. Trends Plant Sci. 2: 379-384.
Lindroth RL and Peterson SS. 1988. Effects of plant phenols on performance of southern armyworm larvae. Oecologia 75: 185-189.
Orians CM, Huang CH, Wild A, Dorfman KA, Zee P, Dao MTT and Fritz RS. 1997. Willow hybridisation differentially affects preference and performance of herbivorous beetles. Entomologia Experimentalis et Applicata 83: 285-294.
Orians CM, Lower S, Fritz RS, and Roche BM. 2003. The effects of plant genetic variatin and soil nutrients on secondary chemistry and growth in a shrubby willow, *Salix sericea:* patterns and constraints on the evolution of resistance traits. Biochem. Systematics and Ecology 31: 233-247.
Tahvanainen J, Julkumen-Tiitto R, and Kettunen J. 1985. Phenolic glycosides govern the food selection pattern of willow feeding beetles. Oecologica 67: 52-56.

Appendices

Table 1

Code	**Species**	**Treatment**
PN 227	*S. matsudana*	light defoliation
PN 229	*S. medemii*	nil
PN 236	*S. glaucophylloides* 'piperi'	nil
PN 249	*S. purpurea*	light defoliation
PN 386	*S. schwerinii*	light defoliation
PN 698	*S. exigua*	nil
PN 741	*S. fragilis*	light defoliation
PN 745	*S. lucida* spp. *lasiandra*	heavy defoliation
PN 751	*S. lasiolepis*	light defoliation
	Kawa	light defoliation

Table 2 Species variation in concentration of selected phenolglycosides

Each data point is a mean of 15 values, three per month from December 2002 – April 2003. Samples were from Aokautere material only.

Species	salicin	salicortin	tremulacin
S. matsudana	2050	3073	2100
S. medemii	310	0	0
S. glaucophylloides 'piperi'	3820	76804	21310
S. purpurea	8620	30121	24900
S. schwerinii	0	45	0
S. exigua	30	0	0
S. fragilis	1640	877	6820
S. lucida spp. *lasiandra*	7740	68941	4760
S. lasiolepis	13100	81626	59870
Kawa	8200	12965	200

Table 3 Geographical variation in selected phenolglycosides

Species	salicin		Chlorogenic acid		tremulacin	
	PN	MA	PN	MA	PN	MA
S. matsudana	2050	1130	310	410	2100	95
S. medemii	310	235	600	190	0	0
S. glaucophylloides 'piperi'	3820	14500	3870	1850	21310	16600
S. purpurea	8620	10500	86	30	24900	4400
S. schwerinii	0	0	496	15	0	0
S. exigua	30	36	410	24	0	0
S. fragilis	1640	2750	1340	570	6820	2350
S. lucida spp. *lasiandra*	7740	38700	350	950	4760	44900
S. lasiolepis	13100	39500	1990	1200	59870	37000
Kawa	8200		1360	1050	200	105

Figure 1 a – c Seasonal variation in selected phenolglycosides

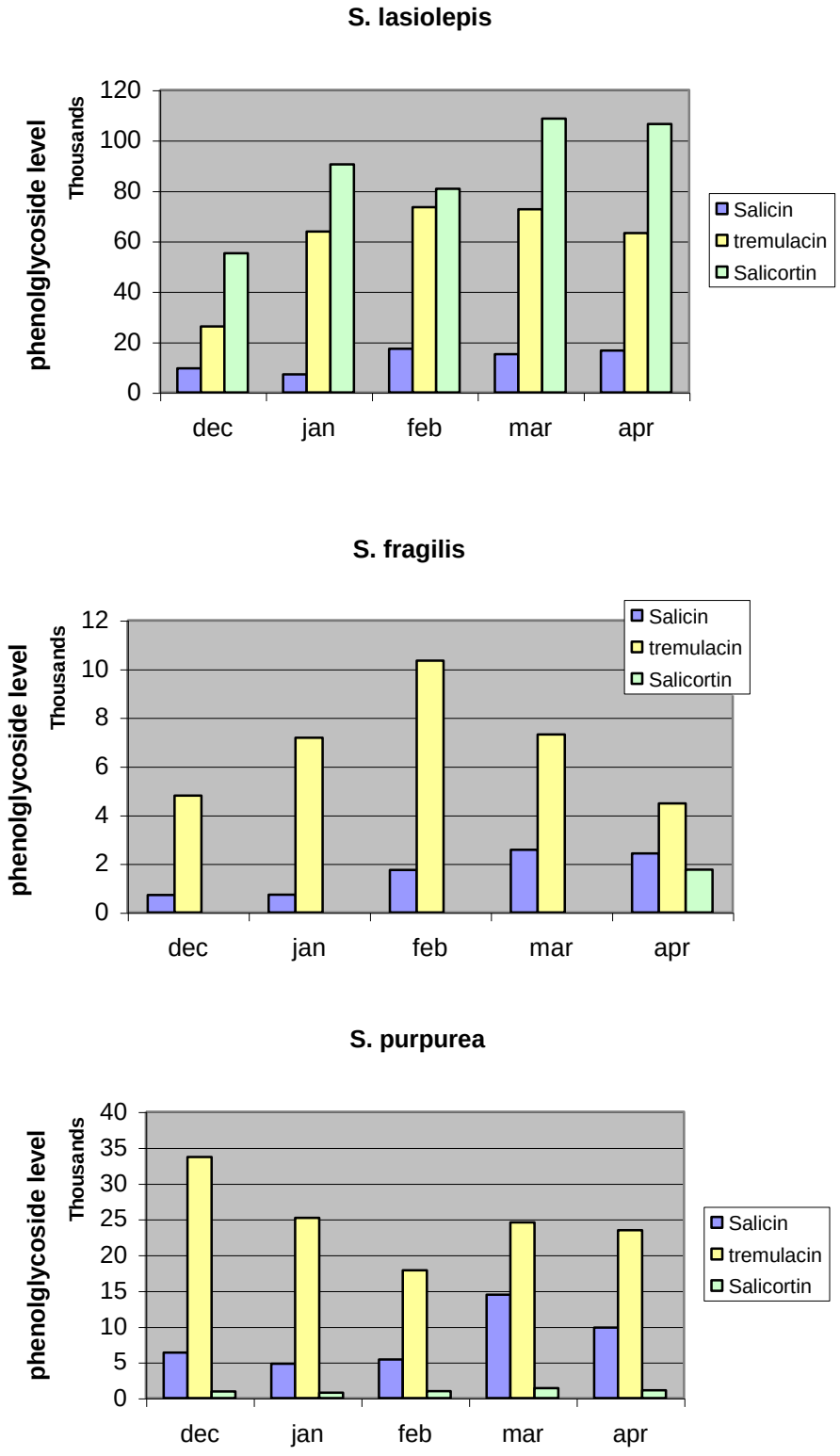

Table 4 Seasonal variation in total phenolglycosides (representative species)

	Species				
Total phenolglycosides	*S. matsudana*	*S. purpurea*	*S. fragilis*	*S. lucida* spp. *lasiandra*	*S. lasiolepis*
dec	22696	22574	9291	10840	4373
jan	30558	50224	18950	12480	24987
feb	31095	61483	23827	9061	24887
mar	24770	78027	27031	9467	31600
apr	59817	73055	33208	7076	55448

Table 5 Analysis of variance in concentrations of salicin, tremulacin and total phenolglycosides following light defoliation (p values). *****absent

Species	**salicin**	**tremulacin**	**Total PGS**
S. purpurea	0.77	0.67	0.18
S. matsudana	0.99	*	0.66
S. schwerinii	*	*	0.47
S. fragilis	0.37	0.99	0.83
S. lasiolepis	0.53	0.62	0.24
Kawa	0.54		0.77

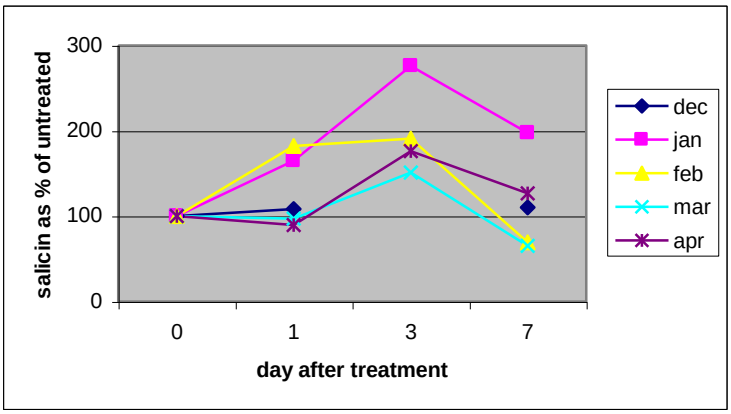

Figure 2 Change in leaf salicin concentration following half leaf defoliation in *S. lasiandra.*

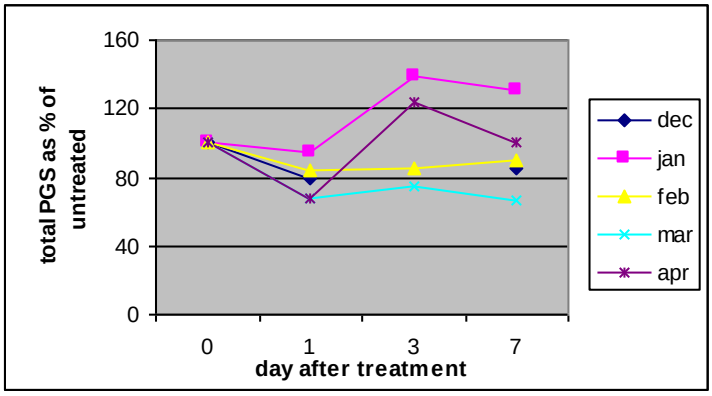

Figure 3. Change in total phenolglycosides following half leaf defoliation in *S. lasiandra*

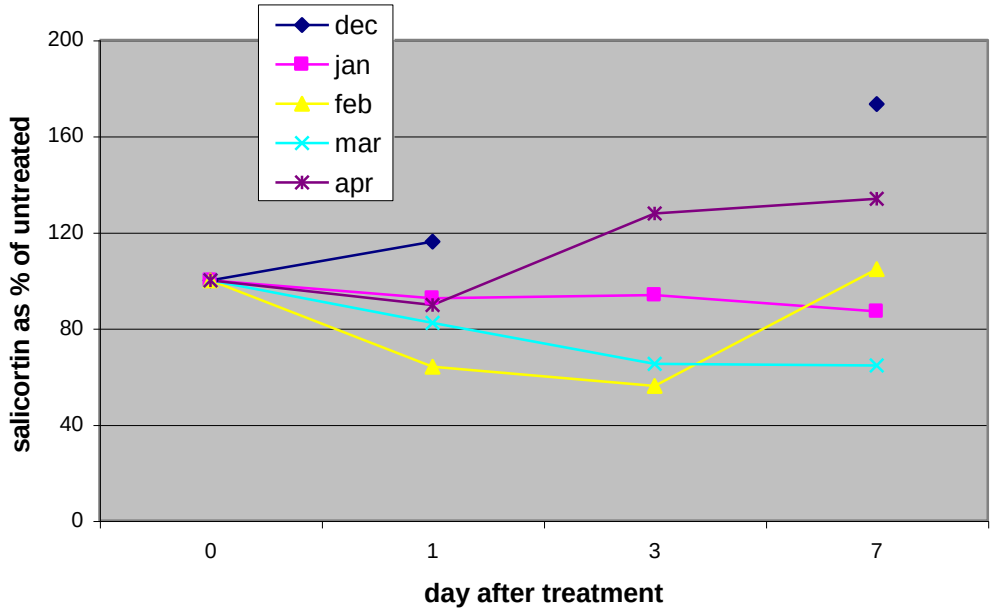

Figure 4. Change in leaf salicortin concentration following half leaf defoliation in *S. lasiandra*

Table 6 Analysis of variance for concentrations of salicin, tremulacin and total phenolglycosides following half leaf (HLD) and whole branch (WBD) defoliation (p values).

Treatment	Salicin	Salicortin	Tremulacin	Total phenolglycosides
HLD	0.02	0.46	0.16	0.21
WBD	0.31	0.83	0.53	0.73