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Salicylates of Intact *Salix myrsinifolia* Plantlets Do Not Undergo Rapid Metabolic Turnover[1]

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Salicylates, the main phenolic glucosides of northern willow (*Salix* spp.), play an important role in plant-herbivore interactions. Salicylates are labile metabolites that are thought to undergo metabolic turnover. Salicylates are synthesized from phenylalanine (Phe) via the shikimate pathway. 2-Aminoindan-2-phosphonic acid (AIP), a strong inhibitor of Phe ammonia-lyase (EC 4.3.1.5), was used to block the biosynthesis of salicylates. The aim of this study was to investigate long-term turnover of salicylates in intact micropropagated plantlets of *Salix myrsinifolia* Salisb. The biosynthesis of salicylates was inhibited efficiently but not completely by 30 μM 2-aminoindan-2-phosphonic acid. Inhibitor treatment, aside from leading to a high accumulation of Phe, also led to an increase in tyrosine and tryptophan, indicating that 2-aminoindan-2-phosphonic acid may also inhibit enzymes other than Phe ammonia-lyase. Salicylates were shown to be unexpectedly stable metabolites that did not undergo marked metabolic turnover in intact plants; in leaves no significant turnover occurred, and in the stems the five salicylates studied were turned over slowly, with half-lives of 11 to 25 d. The total amount of salicylate in mature shoots decreased only 0.6% per day.

The phenolic glucosides of the Salicaceae play an important role in plant-herbivore interactions, which have been studied intensively by many research groups. These glucosides may protect plants against many generalist insect herbivores (Tahvanainen et al., 1985b; Lindroth et al., 1988; Lindroth and Peterson, 1988; Clausen et al., 1989; Denno et al., 1990). But on the other hand, they may act as positive cues or feeding stimulants for specialist herbivores (Matsuda and Matsuo, 1985; Smiley et al., 1985; Tahvanainen et al., 1985b; Rowell-Rahier and Pasteels, 1986; Denno et al., 1990; Rank, 1992). Some specialists may use phenolic glucosides for their own defense against predators (Pasteels et al., 1983; Smiley et al., 1985; Rowell-Rahier and Pasteels, 1986; Denno et al., 1990). Phenolic glucosides also affect the food selection of mammalian herbivores (Palo, 1984; Smiley et al., 1985; Tahvanainen et al., 1985a; Reichardt et al., 1990; Bryant et al., 1992) and are considered to be constitutive defenses because they are present in plant tissues all the time (Feeney, 1976).

The ability to synthesize a variety of simple phenolic glucosides is characteristic for willows (*Salix* spp.) and other members of the Salicaceae family (Thieme, 1965c; Egloff, 1982; Palo, 1984; Julkunen-Tiitto, 1989). Salicylates, a group of chemically related phenolic glucosides based on the structure of salicin (Fig. 1), are the most common phenolic glucosides found in willows (Palo, 1984; Julkunen-Tiitto, 1989). Salicin and salicortin are the most widespread salicylates (Palo, 1984; Julkunen-Tiitto, 1989), while acetylsalicortin is found in only a few willow species (Julkunen-Tiitto, 1989).

Salicortin and other salicylates such as tremulacin and acetylsalicortin, which contain a 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety, are very labile in vitro and easily degrade to salicin (Thieme, 1965b; Pearl and Darling, 1971; Lindroth and Pajutee, 1987; Meier, 1988) and then to saligenin (Julkunen-Tiitto and Tahvanainen, 1989) during the isolation procedure (Fig. 1). At the same time, the labile 1-hydroxy-6-oxo-2-cyclohexen-1-carbonyl moiety is released and converted to 6-hydroxy-2-cyclohexenone (6-HCH) and catechol (Julkunen-Tiitto and Meier, 1992b), which are effective components of salicortin and its derivatives in defense against herbivores (Clausen et al., 1989; Reichardt et al., 1990). Improper preservation of samples (Julkunen-Tiitto, 1985; Lindroth and Pajutee, 1987; Julkunen-Tiitto and Gebhardt, 1992; Orians, 1995), long extraction times (Julkunen-Tiitto, 1985), and freezing (Julkunen-Tiitto, 1989) may all cause the degradation of salicortin.

As carbon-based compounds that do not contain nitrogen, the salicylates are thought to be quite cheap defenses for plants, especially when nitrogen is the growth-limiting factor (Bryant et al., 1983). On the other hand, in certain willow species the concentrations of salicylates may be very high (Julkunen-Tiitto, 1989), and if they also undergo rapid metabolic turnover (Reichardt et al., 1991), their maintenance at a given concentration may demand large amounts of resources from the plants (Gershenzon, 1994).

Phenolic glucosides of Salicaceae are considered to be "dynamic" substances that are subjected to metabolic turnover (Reichardt et al., 1991), but no earlier studies have determined the rate of metabolic turnover. According to models used to estimate the metabolic costs of the chemical defense of plants (Fagerström, 1989; Skogsmyr and Fagerström, 1992), an increase in concentration of the defense substance or its metabolic turnover rate raises the cost of defense.

The aim of this work was to ascertain whether willow salicylates are exposed to metabolic turnover. Because of the high inter-individual variation in the concentrations of salicylates (Julkunen-Tiitto, 1985, 1989; Meier, 1988; Julkunen-Tiitto and Meier, 1992a), clonal material was used in the experiment. *Salix myrsinifolia* was chosen because it

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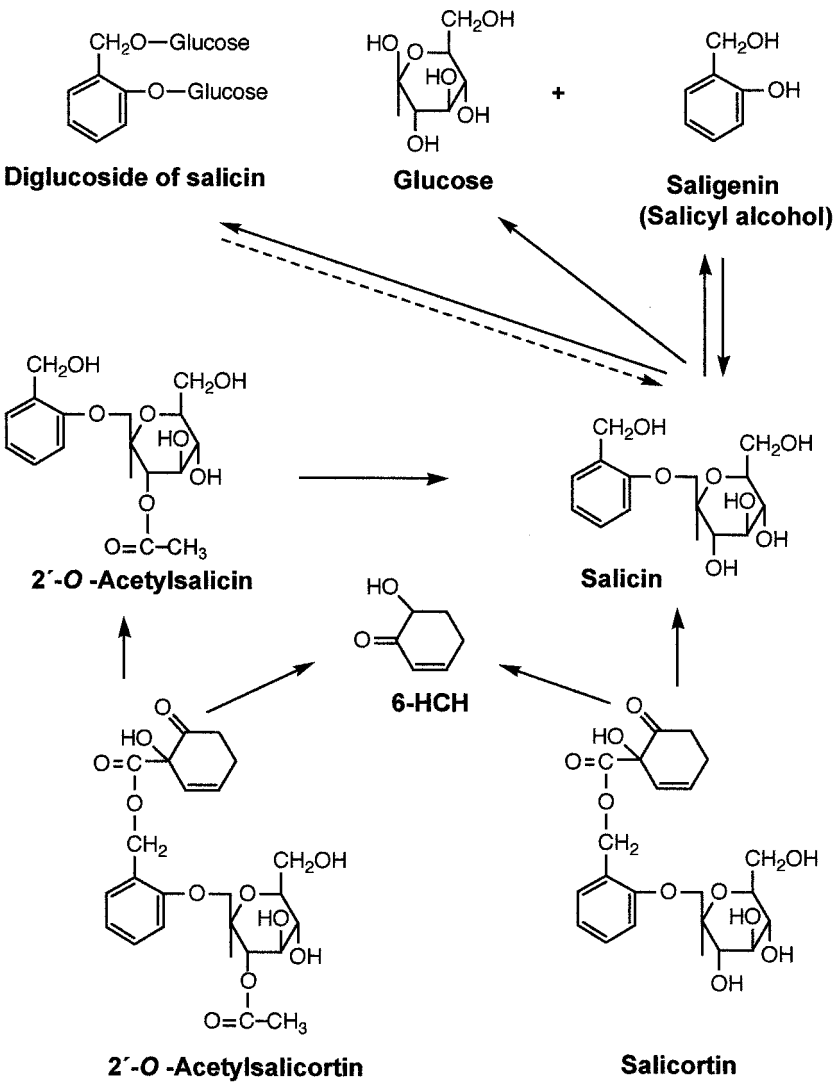

Figure 1. Chemical structures of salicylates of *S. myrsinifolia* and their interrelationships. Salicortin degrades to salicin and 2′-*O*-acetylsalicortin to 2′-*O*-acetylsalicin and further to salicin and in both cases a toxic 6-HCH-moiety will be released. 2′-*O*-acetylsalicin was not detected in this study. Salicin will degrade to saligenin (salicyl alcohol) by the hydrolytic removal of a Glc molecule. Saligenin can be converted back to salicin and salicin to diglucoside of salicin by glucosylation. The dashed line from diglucoside of salicin to salicin indicates a hypothetical step.

contains a high level of salicylates, especially salicortin, and is easy to culture in vitro. In addition, *S. myrsinifolia* is one of the most promising willow species for the production of herbal drugs (Julkunen-Tiitto and Meier, 1992a), so it is important to ascertain to what extent the maintenance of a high level of salicylates requires plants resources

MATERIALS AND METHODS

Plant Material

We used *Salix myrsinifolia* Salisb. (clone V8) plantlets cultured in vitro (Julkunen-Tiitto, 1996) for the experiment. Fresh plant material was used in all analyses. Mature leaves, stems, and shoot apexes with two to four upper leaves were analyzed separately. Because the roots of this species do not contain salicylates, they were not analyzed.

Experimental Procedure

Six-week-old rooted plantlets (4–6 cm high) that had been cultured in solidified Murashige and Skoog medium (Murashige and Skoog, 1962) were transferred aseptically into culture vessels on cotton pads moistured with 15 mL of liquid Murashige and Skoog medium. The Suc content of the growth medium was 3% (w/v) and no hormones were added. The plantlets were precultivated for 5 d to allow them to root into the cotton pads. After precultivation, the plant material was divided into two treatments: (a) control plants without inhibitor and (b) plants treated with 30 μM 2-aminoindan-2-phosphonic acid (AIP). The inhibitor was first dissolved in water, and aqueous AIP (10 mM) was mixed into liquid Murashige and Skoog medium. Growth medium (5 mL) with 120 μM AIP was added to one-half of the culture vessels so that the final volume of the growth medium was 20 mL and the AIP concentration was 30 μM. To the other half of the vessels (the control plants), 5 mL of Murashige and Skoog medium without inhibitor was added.

The experiment lasted 10 d. Samples were taken on the 1st d (d 0) and after that every 2nd d. Roots were removed from the plantlets, and the shoot apex with two to four upper leaves and mature leaves were cut and weighed.

Stems were analyzed with petioles. The plantlets in one culture vessel were pooled and analyzed together. One plantlet per culture vessel was used for determination of dry weight after overnight drying at 105°C.

After preparation and weighing, the fresh samples were immediately put into cold methanol on an ice bath for 15 to 20 min. Samples were cut into small pieces (in methanol) with scissors and then extracted twice (2 × 2 min) with methanol (2 × 10 mL for leaves and stems, 2 × 5 mL for shoot tips) using a homogenizer (Ultra-Turrax, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and then filtered. Methanol was reduced to dryness in a vacuum evaporator; the residues of leaf and stem samples were redissolved in 10 mL of methanol and residues of shoot tip samples in 6 mL of methanol. Samples were divided into 1-, 2-, and 3-mL aliquots, and methanol was evaporated to dryness with gaseous nitrogen. Samples were stored at −20°C. Before HPLC analysis, the samples were redissolved in dH_2O/methanol (1:1, v/v).

HPLC Analysis

The samples were analyzed by HPLC (Hewlett-Packard, Palo Alto, CA), which consisted of quaternary solvent delivery and an autosampler system (HP 1050 Series). The compounds were separated on a 60- × 4.6-mm i.d. HP Hypersil ODS II (3-μm) column. A photodiode array detector (HP 1040A Series) coupled to a data system/personal computer (Hewlett-Packard), was used to record chromatograms and UV-Vis spectra. The elution conditions and gradient used for salicortin, 2′-*O*-acetylsalicortin, saligenin, diglucoside of salicin, and Trp were as described by Julkunen-Tiitto et al. (1996b). The elution solvents for salicin, Phe, and Tyr were A (0.5% [v/v] tetrahydrofuran and 0.21% [v/v] *O*-phosphoric acid in dH_2O) and B (100% [v/v] methanol). The samples were eluted according to the following gradient: 0 to 5 min, 100% of A; 5 to 7 min, 0% to 100% of B in A; 7 to 12 min 100% of B; and 12 to 13 min, 100% of A. The flow rate was 2 mL/min.

Identification and Quantification of Compounds

Compounds were identified by comparing their retention times and spectral characters to those of the reference compounds. The analyzed compounds were quantified at 220 nm. The compounds were quantified as follows: salicin and diglucoside of salicin were based on salicin; salicortin and 2′-*O*-acetylsalicortin were based on salicortin; and saligenin was based on saligenin as a reference compound. Phe, Tyr, and Trp were based on commercial compounds.

Calculations and Statistics

The experiment was repeated three times. In the first two experiments there were two replicates per sampling point, and in the third there were two replicates at d 0 and one replicate at the rest of the sampling points. Therefore, the results are the mean of six (d 0) or five (the rest of the sampling points) replicates. A culture vessel containing five plants analyzed together was considered to be one replicate.

The changes in the salicylate concentrations during the experiment followed first-order kinetics. Data were *ln*-transformed and evaluated by linear regression and differences between regression coefficients of control and AIP lines were tested with Student's *t* test (Zar, 1999). Half-lives of salicylates were calculated by an equation of first-order kinetics. Amino acid concentrations were first changed to relative values by comparing the concentrations in AIP-treated plants to the mean concentrations in control plants. Relative concentrations were then evaluated by Spearman's nonparametric correlation. Inhibition percentages of the biosynthesis of salicylates were calculated by comparing relative values (calculated as above) at d 0 to the concentrations at d 10. Differences in the total salicylate contents between different parts of plant at d 0 were tested by univariate analysis of variance and Tukey's HSD test. All statistical programs were standard versions of SPSS (1997) for Windows 8.0 (SPSS, Chicago).

RESULTS

Distribution of Phenolics in Different Parts of Plants

The most abundant phenolics in micropropagated *S. myrsinifolia* were salicylates (data not shown). The highest content of total salicylates was found in the young shoot tips: over 10% (w/w) on a dry weight basis. The mature leaves contained about 6% (w/w) salicylates and the stems under 3% (w/w) on a dry weight basis (Fig. 2A). The distribution of total salicylates differed significantly between plant parts (Fig. 2). In all plant parts, salicortin was the most abundant salicylate (Fig. 2, B–D). In the leaves the amount of salicortin was 62% (Fig. 2C), in the stems 76% (Fig. 2B), and in the shoot tips 68% of the total salicylate content (Fig. 2D). The relative amount of salicin was the same in all plant parts (12%–14%), but the amount of 2′-*O*-acetylsalicortin varied according to the part: in the stems the proportion of acetylsalicortin was only 8%, but in the leaves it was as high as 24%, and in the shoot tips 17%. The levels of saligenin and diglucoside of salicin were low in whole shoot (0.7%–2.2%).

Inhibition of Biosynthesis of Salicylates in the Shoot Tips

Low-*M*r glucosides of willows are suggested to be derived from trans-cinnamic acid (Zenk, 1967), which is synthesized from Phe by a deamination reaction catalyzed by Phe ammonia-lyase (PAL; EC 4.3.1.5.). AIP, a specific and powerful inhibitor of PAL (Zón and Amrhein, 1992), was used to block the biosynthesis of cinnamic acid derivatives.

A problem encountered when the long-term turnover study was planned was that the willows grow vigorously, and the remaining concentrations of secondary metabolites in the inhibitor-treated plants would be diluted by the increasing volume of tissue during the experiment. To minimize this dilution effect, the stem segments above the first mature leaf were marked at the beginning of the exper-

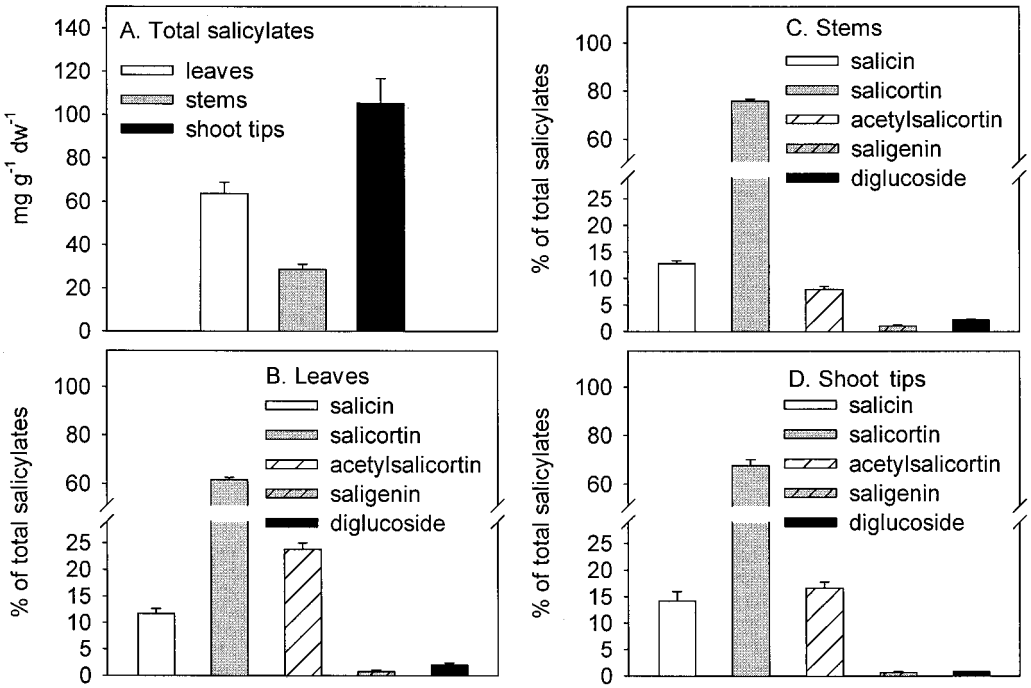

Figure 2. Total amount of salicylates in the leaves, stems, and shoot tips at the beginning of the experiment (A), and the relative amounts of five salicylates in the leaves (B), stems (C), and shoot tips (D) of micropropagated *S. myrsinifolia* plantlets. The results are the means of six samples; bars indicate SE. The total amounts of salicylates in the different parts of plants were tested by univariate analysis of variance and Tukey's HSD test. Differences were significant between all plant parts (leaves and stems, $P \leq 0.05$; leaves and shoot tips, $P \leq 0.01$; stems and shoot tips, $P \leq 0.001$).

iment, so that the new shoot parts that grew after the addition of the inhibitor could be separated from these leaves and stems that were mature before the addition of the inhibitor. Turnover rates were determined from mature leaves and stems. Percentages of inhibition of biosynthesis of salicylates were determined from shoot tips.

At the beginning of the experiment, the shoot tips of the AIP-treated plantlets were very rich in salicylates, but when the shoots grew and new apexes developed, the concentration of salicylates decreased dramatically, indicating effective inhibition of their biosynthesis by AIP treatment (Fig. 3). The concentrations of salicin, salicortin, and 2′-*O*-acetylsalicortin in the control shoot tips remained constant during the experiment (Fig. 3, A–C), but the concentration of diglucoside of salicin decreased significantly (Table I; Fig. 3E) and the level of saligenin decreased slightly but not significantly (Table I; Fig. 3D) in the control shoot tips. On the last day of experiment (d 10), the shoot tips were entirely new tissue that had grown after the addition of 30 μM AIP. When the relative concentrations of salicylates on the first (d 0) and last (d 10) day were compared, the biosynthesis of salicortin was inhibited 78% (w/w), 2′-*O*-acetylsalicortin 73% (w/w), and salicin 71% (w/w). Inhibition of the biosynthesis of the minor salicylates saligenin and diglucoside of salicin was less effective: 52% and 36%, respectively.

The inhibitor treatment had no visible effect on the growth or health of the plantlets. The average dry weight of stems or the leaf mass used for the analyses did not increase significantly during the experiment, indicating that the mature parts of micropropagated plantlets did not expand markedly, and thus the dilution of salicylate concentrations by growth was minimal. We did not measure the elongation rate of the shoots.

Accumulation of Aromatic Amino Acids in the AIP-Treated Plantlets

Extensive accumulation of Phe in the inhibitor-treated plants during the experiment confirmed that AIP inhibited PAL effectively (Fig. 4, A–C). Accumulation was most intensive in the young shoot tips and least intense in the mature leaves. In the shoot tips, the concentration of Phe was highest on d 10 (70.3 ± 10.8 μmol g^{-1} dry $weight^{-1}$). In the stems and leaves the concentration reached its maximum on d 8 (51 ± 8.9 and 30.1 ± 5.9 μmol g^{-1} dry $weight^{-1}$, respectively), after which it declined.

No Phe was detected in the chromatograms of the control plants, but a slight amount of Tyr was present. In the mature leaves (Fig. 4D) and the young shoot tips (Fig. 4F), the amount of Tyr increased significantly. In the stems, the increase in Tyr and Trp was not significant (Fig. 4E and G). In shoot tips (Fig. 4I) of AIP-treated plants, the amount of Trp increased markedly and significantly.

Half-Lives and Turnover Rates of the Salicylates

In mature leaves, all salicylates were stable (Fig. 5); no significant turnover occurred in the salicylate pools (Table I). The concentrations of salicortin, 2′-*O*-acetylsalicortin,

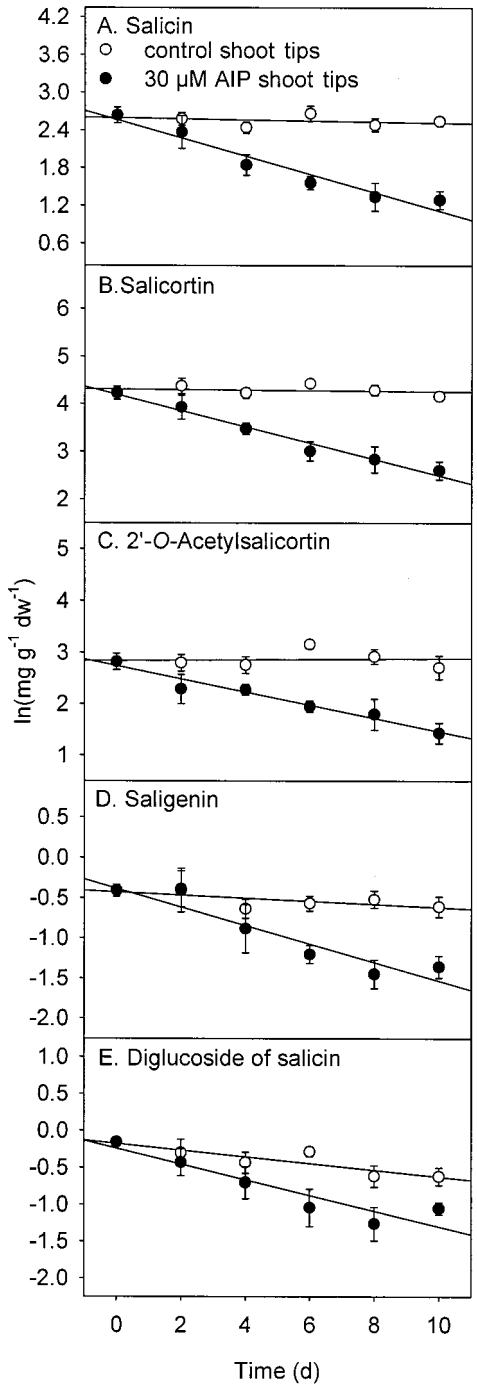

Figure 3. Levels of salicin (A), salicortin (B), 2′-*O*-acetylsalicortin (C), saligenin (D), and diglucoside of salicin (E) in the shoot tips of control and AIP-treated plants during the turnover study. Shoots grew during the experiment, producing new apexes. The shoot tips of control plants were rich in salicylates during the whole experiment, whereas newly produced shoot tips of AIP-treated plants had a low salicylate content due to inhibition of biosynthesis of cinnamic acid derivatives. Results are the mean of five to six samples; bars indicate ±SE. dw, Dry weight.

and diglucoside of salicin in the leaves of the AIP-treated plantlets decreased significantly, but since the concentrations also decreased slightly in the control leaves (Fig. 5, B, C, and E), the difference in regression coefficients was not significant (Table I). The concentrations of salicin and saligenin during the experiments did not change significantly in either the control or the AIP-treated leaves (Fig. 5, A and D; Table I).

Unlike mature leaves, in the stems the concentrations of all five salicylates decreased slowly but significantly (Fig. 6; Table I). The half-life of the diglucoside of salicin was the shortest (10.5 d), and the half-lives of salicin and its degradation product, saligenin, were longest (25.3 and 21.9 d, respectively) (Table II). The half-lives of the most labile salicylates, salicortin and 2′-*O*-acetylsalicortin, were also surprisingly long (15.0 d) (Table II).

During the experiment, the concentrations of salicin and saligenin did not decrease significantly in the AIP-treated stems, but since their concentrations increased slightly in the control stems (Fig. 6, A and D), the differences in regression coefficients were significant (Table I). In the control stems, the level of diglucoside of salicin also decreased significantly, but the decrease was more pronounced in the AIP-treated stems (Fig. 6E) and the difference in coefficients was significant (Table I).

The turnover rate of the most abundant salicylate, salicortin, was the most rapid, 8.20 nmol d^{-1} $stem^{-1}$, representing the largest individual salicylate pool that needed resynthesis (Table II). The turnover rate of salicin was faster than that of 2′-*O*-acetylsalicortin (1.25 and 0.75 nmol d^{-1} $stem^{-1}$, respectively). The turnover rates of the minor salicylates, saligenin and diglucoside of salicin, were the slowest (Table II). When the turnover rates of salicylates per stem were calculated together, a decrease of 10.8 nmol per day (Table II) represents 0.6% of the total salicylate content of mature shoots (1,830 nmol). In the other words, the salicylate content of mature shoots of micropropagated willow plantlets decreased only 0.6% per day.

DISCUSSION

Salicylates of Intact Willow Plantlets Do Not Undergo Rapid Turnover

In spite of the fact that salicortin and 2′-*O*-acetylsalicortin are labile in vitro in intact *S. myrsinifolia* plantlets, these compounds were shown to be unexpectedly stable in vivo. In the mature leaves no turnover occurred, and in the stems the half-lives were long and the turnover rates slow. The turnover rate of total salicylates was as slow as 10.8 nmol d^{-1} mature $shoot^{-1}$. For example, the turnover rate of dhurrin in intact, green *Sorghum bicolor* seedlings was 4.8 nmol h^{-1} $shoot^{-1}$, which corresponds to a turnover rate of 115.2 nmol d^{-1} $shoot^{-1}$ (Adewusi, 1990). The total amount of salicylates in the mature shoots decreased only 0.6% per day. For example, Thieme (1965a) observed 20% and 40% diurnal variation in the total phenolic glucoside content of the leaves of two central European willows, *Salix fragilis* and *Salix purpurea*. Such a high variation could be the result of even slightly inaccurate sampling of willow leaves, since according to present knowledge there is a high variation in the concentration of salicylates according to the develop-

Table I. *Test results of linear regression and comparison of coefficients of the control and AIP lines*

Changes in the concentrations of salicylates of control and AIP-treated plants during the turnover study were tested by linear regression after *ln*-transformation. Differences between regression coefficients of control and AIP-treated lines were tested by Student's *t* test ($n = 29–30$).

Plant Part and Compound	Regression Coefficients				Comparison of Coefficients	
	Control		AIP		*t*-Value	
Shoot tips						
Salicin	-8.6×10^{-3}	ns[a]	−0.144	***[b]	6.716	***
Salicortin	-4.9×10^{-3}	ns	−0.170	***	6.855	***
Acetylsalicortin	-3.6×10^{-3}	ns	−0.128	***	4.479	***
Saligenin	-1.9×10^{-2}	ns	−0.115	***	3.731	***
Diglucoside	-4.5×10^{-2}	**[c]	−0.106	***	2.419	*[d]
Leaves						
Salicin	3.3×10^{-3}	ns	2.6×10^{-3}	ns	4.1×10^{-2}	ns
Salicortin	-6.4×10^{-3}	ns	-2.2×10^{-2}	*	1.107	ns
Acetylsalicortin	-3.7×10^{-3}	ns	-2.1×10^{-2}	***	1.993	ns
Saligenin	6.8×10^{-3}	ns	1.4×10^{-2}	ns	0.500	ns
Diglucoside	-7.8×10^{-3}	ns	-3.0×10^{-2}	**	1.385	ns
Stems						
Salicin	2.1×10^{-2}	ns	-2.8×10^{-2}	ns	2.721	**
Salicortin	-1.1×10^{-2}	ns	-4.6×10^{-2}	***	2.457	*
Acetylsalicortin	-2.2×10^{-3}	ns	-4.6×10^{-2}	**	2.122	*
Saligenin	1.8×10^{-2}	ns	-3.2×10^{-2}	ns	2.384	*
Diglucoside	-2.3×10^{-2}	*	-6.6×10^{-2}	***	2.924	**

[a] ns, Non-significant. [b] $***P \leq 0.001$. [c] $**P \leq 0.01$. [d] $*P \leq 0.05$.

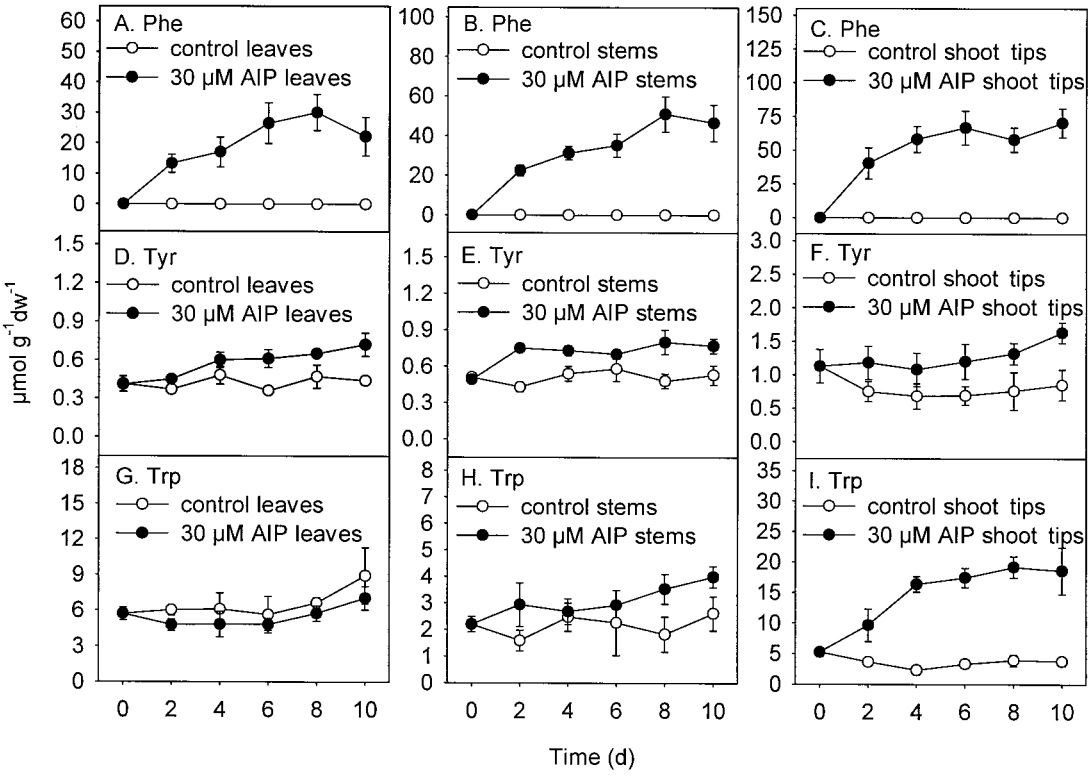

Figure 4. Accumulation of Phe (A–C), Tyr (D–F), and Trp (G–I) in the leaves (A, D, and G), stems (B, E, and H), and shoot tips (C, F, and I) during the turnover study. Phe accumulated significantly in all three plant parts in AIP-treated plants ($P \leq 0.001$). Accumulation of Tyr was significant in the leaves ($P \leq 0.001$) and shoot tips ($P \leq 0.01$) of AIP-treated plants, and accumulation of Trp in the shoot tips ($P \leq 0.01$). Results are the mean of five to six samples; bars indicate ±SE. Relative concentrations of amino acids (treated/control) were evaluated by Spearman's nonparametric correlation. dw, Dry weight.

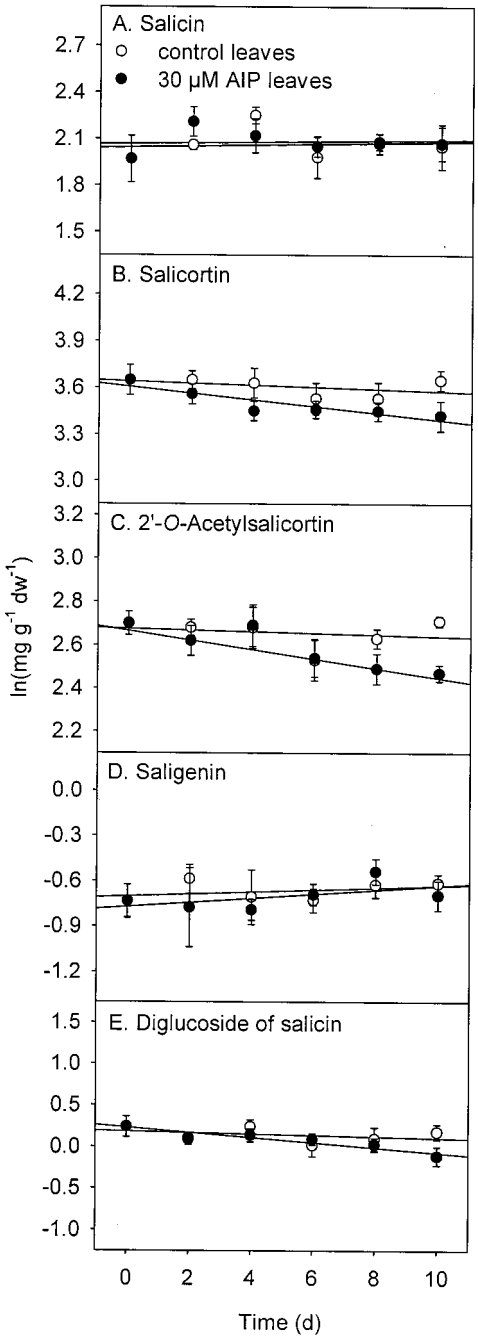

Figure 5. The concentrations of salicin (A), salicortin (B), 2′-*O*-acetylsalicortin (C), saligenin (D), and diglucoside of salicin (E) in mature leaves of control plants and plants treated with 30 μM AIP during the turnover study. Data were *ln*-transformed before linear regression. Results are the mean of five to six samples; bars indicate ±SE. dw, Dry weight.

ment stage of the leaf and/or the position of the leaf in the shoot (Julkunen-Tiitto, 1989).

One explanation for the lack of rapid turnover in our experiment could be that we used intact plants. In most turnover studies, excised plants or plant parts are used. According to Gershenzon (1994), high turnover rates are in many cases artificial due to detachment of plant parts. Mihaliak et al. (1991) demonstrated that rapid turnover of monoterpenes in peppermint was the result of shoot detachment and does not occur in normal physiological conditions. On the other hand, in intact pine seedlings coniferin turned over with a half-life of 60 to 120 h, but coniferin may be an intermediate in lignin biosynthesis, and thus the

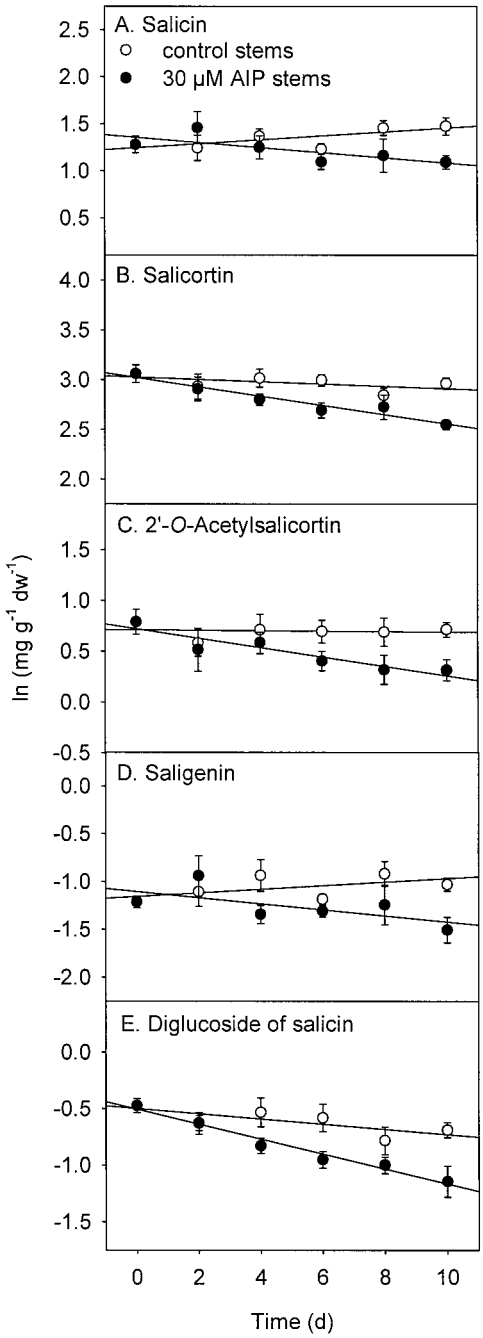

Figure 6. Concentrations of salicin, salicortin, 2′-*O*-acetylsalicortin, saligenin, and diglucoside of salicin in the stems of control plants and plants treated with 30 μM AIP during the turnover study. Data were *ln*-transformed before linear regression. Results are the mean of five to six samples; bars indicate ±SE. dw, Dry weight.

Table II. *Half-lives and turnover rates of salicylates in the stems of S. myrsinifolia*

Regression coefficients were determined by linear regression after the data were *ln*-transformed, and half-lives of salicylates were calculated using the equation of first-order kinetics.

Salicylate	$t_{1/2}$[a]	Concentration at D 0		Turnover Rate	
	d	$\mu mol\ g^{-1}\ dry\ wt^{-1}$	$nmol\ stem^{-1}$	$nmol\ d^{-1}\ g^{-1}\ dry\ wt^{-1}$	$nmol\ d^{-1}\ stem^{-1}$[b]
Salicin	25.3	12.8 ± 1.1	63.0	253	1.25
Salicortin	15.0	49.3 ± 4.3	246	1,640	8.20
Acetylsalicortin	15.0	4.59 ± 0.6	22.6	153	0.753
Saligenin	21.9	2.42 ± 0.2	11.8	110	0.269
Diglucoside	10.5	1.40 ± 0.1	6.89	66.7	0.328
Total		70.5 ± 6.3	350	2,230	10.8

[a] $t_{1/2} = \ln 2/k_d$ where $t_{1/2}$ = half-life, k_d = regression coefficient. [b] Turnover rates were calculated using the mean dry weight of stems (4.92 mg).

reduction could be due to conversion of coniferin to lignin (Marcinowski and Grisebach, 1977). Amrhein and Diederich (1980) showed that two isoflavones of intact *Cicer arietinum* L. plants are differentially turned over: biochanin A had a very low (or no) turnover, but formononetin was turned over with a half-life of 72 h. Furthermore, they demonstrated that inhibitors of PAL can be used in turnover studies of cinnamic acid derivatives.

Kleiner et al. (1999) showed by using $^{14}CO_2$ that phenolic glycosides of *Populus tremuloides* (Salicaceae) decreased by 58% within 48 h of labeling, which indicates a high turnover rate. The weakness of this otherwise carefully designed experiment was that they used only three plants, which were wounded and defoliated during the experiment, and they compared only two sampling points (24 and 48 h). If the whole period of chase hours (168 h) had been taken into account, no significant turnover would have occurred in the pool of phenolic glycosides of source leaves. Moreover, they did not observe significant changes in total phenolics or condensed tannins during the experiment. If the level of phenolic glycosides had declined markedly, this should have been reflected in the level of total phenolics. In our subsequent study, in which *Salix pentandra* plantlets grew for 3 weeks in the presence of AIP, the amount of salicylates did not decrease markedly in the mature leaves; only the level of salicin decreased significantly (T.M. Ruuhola and M.-R.K. Julkunen-Tiitto, unpublished data). As in *S. myrsinifolia* and *S. pentandra*, the salicylates of stems were turned over slowly (T.M. Ruuhola and M.-R.K. Julkunen-Tiitto, unpublished data).

However, turnover rates of salicylates might be different in in vitro cultured plantlets than in nature. Neither do we know how AIP treatment affects the natural turnover rates of salicylates, since the inhibition of such important enzyme as PAL creates unnatural conditions in cells, which may have further effects on the activity of other enzymes. In addition, the possibility that salicylates are translocated from stems into the growing shoot tips cannot be ruled out. The fact that the salicylate content of the shoot tips of AIP-treated plantlets did not decrease under the concentrations observed in the stems may indicate that the turnover of salicylates in the stems could be partly due to translocation of salicylates into developing shoot tips with very low salicylate content.

Inhibition of the Biosynthesis of Salicylates by AIP and Accumulation of Three Aromatic Amino Acids

In young, developing shoot tips, the biosynthesis of salicylates was inhibited effectively but not completely by 30 μM AIP. AIP is the most effective PAL inhibitor known in vivo (Zón and Amrhein, 1992). For example, the synthesis of caffeic acid esters was completely inhibited by AIP (Schmutz et al., 1992), and the biosynthesis of 5-caffeoylquinic acid was inhibited by 85% (Mösli Waldhauser and Bauman, 1996).

In our experiment, inhibition of the biosynthesis of the major salicylates, salicortin, salicin, and 2′-*O*-acetylsalicortin, was more pronounced than inhibition of the biosynthesis of the minor salicylates, saligenin and diglucoside of salicin, probably because these substances could be produced from salicin by the hydrolytic removal of glucoside or glucosylation, respectively (Julkunen-Tiitto et al., 1996a). Salicin is, in turn, easily produced by degradation of salicortin and 2′-*O*-acetylsalicortin (Fig. 1); therefore, the biosynthesis of salicin, saligenin, or diglucoside of salicin cannot be completely blocked as long as salicortin or its derivatives are present in the tissue.

In our subsequent study, the exogenous application of *t*-cinnamic acid increased significantly the amount of diglucoside of salicin, 2′-*O*-acetylsalicortin, and two other acetylated salicortins in the shoot tips of AIP-treated *S. pentandra* plantlets (T.M. Ruuhola and M.-R.K. Julkenen-Tiitto, unpublished data), which confirms that the reduction of salicylate concentration by AIP treatment is due to the inhibition of PAL. The application of *t*-cinnamic acid did not, however, restore the salicylate content to the level observed in the AIP controls. It is very likely that *t*-cinnamic acid was primarily used for the lignification of new plant parts.

The large accumulation of Phe in the inhibitor-treated plantlets further verified that PAL was effectively inhibited by AIP. A large accumulation of Phe has been observed in several studies in which L-α-aminooxy-β-phenyl propionic acid (AOPP) was used as a PAL inhibitor (Amrhein and Holländer, 1979; Holländer et al., 1979; Nóe et al., 1980; Havir, 1981; Amrhein et al., 1983; Holländer-Czytko and Amrhein, 1983). The level of Tyr also increased, which may indicate that Tyr ammonia-lyase (TAL) of *S. myrsinifolia*

was inhibited by AIP, since AOPP also blocks the function of TAL (Holländer et al., 1979) and Tyr decarboxylase (EC 4.1.1.25) (Chapple et al., 1986). TAL catalyzes the deamination of Tyr, producing *p*-coumaric acid directly (Strack, 1997). Instead, Phe must first be converted to *t*-cinnamic acid, which is in turn hydroxylated to *p*-coumaric acid in the biosynthesis of phenylpropanoids. The most unexpected result was the accumulation of Trp, which has not been reported in earlier inhibitor studies. Trp is also produced via the shikimate pathway and many secondary metabolites are produced from Trp, e.g. simple amine derivatives and alkaloids (Seigler, 1998), which have not, however, been shown to be found in willows.

Even though we did not measure elongation rates of control and AIP-treated plantlets, it was clear that AIP treatment did not have any visible effects on the health or growth of the plantlets. The high accumulation of aromatic amino acids supports this observation. One reason AIP treatment did not benefit growth could be that there is some other factor that restricts growth, e.g. inhibition of lignification of new tissue and disturbance of formation of normal xylem vessels, as shown by Amrhein et al. (1983). They reported that growth over 9 d in the presence of AOPP had a little effect on fresh weight or axis length of the shoots of mungbean. However, when they grew seedlings for a longer period in the presence of 0.7 or 1.0 mM AOPP, the growth ceased and seedlings wilted and eventually died. In the presence of 0.3 mM AOPP the fresh weight increased but stem elongation was reduced 50%. We observed a similar but stronger effect with AIP: if *S. myrsinifolia* plantlets were transferred at the age of 1 week into growth medium containing 30 μM AIP, the elongation of stems was inhibited almost completely and plantlets formed rosettes.

Developmental-Stage-Dependent Accumulation of Phe and Salicylates

S. myrsinifolia belongs to the salicylate-rich willows that have a high salicylate content in their leaves (Julkunen-Tiitto, 1986). Willows grow indeterminately, producing new shoot parts during the whole growth period. Thus, in addition to its use for growth and lignification of developing vascular tissue, a rather large part of Phe is also used for the production of soluble salicylates.

The highest concentration of salicylates in micropropagated *S. myrsinifolia* plantlets was found in the young shoot tips of control plants (over 10% of dry weight, w/w). This is consistent with the most intensive accumulation of Phe in the shoot tips of AIP-treated plants, in which the biosynthesis of phenylpropanoids and salicylates was inhibited. Tyr and Trp also accumulated most intensively at the shoot tips. In the fully expanded mature leaves, where salicylate content was the most stable, the accumulation of Phe was the lowest; however, in the stems, where only a slight turnover of salicylates was observed, the accumulation of Phe was more intensive. Our results are consistent with those of Subramaniam et al. (1993), who in hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) observed the highest PAL expression in the young stems, apical buds, and young leaves. Expression was lower in older stems and undetectable in mature leaves. In non-vascular tissue of the leaves and stems, they detected high PAL expression, which disappeared during maturation. They suggested that non-vascular PAL expression was related to the biosynthesis of soluble phenolic glucosides found in high concentrations in the young parts of poplar.

In our earlier experiment the phenolic glucoside content of *S. purpurea* and *S. myrsinifolia* was shown to be higher in young leaves than in mature leaves (Julkunen-Tiitto, 1989). In addition, juvenile twigs of *Salix caprea*, *Salix nigricans*, and *Salix phylicifolia* contained more phenolic glucosides than did mature twigs, which mountain hares *(Lepus timidus*) preferred over juvenile ones (Tahvanainen et al., 1985a). Similarly, Kleiner et al. (1999) demonstrated that the concentration of total phenolics was twice as great and condensed tannins were 1.7 times greater in the sink than in the source leaves of *Populus tremuloides*. Opposite results have been reported by Denno et al. (1990), who found that the mature leaves of *Salix dasyclados* and *S. fragilis* contain more salicylates and phenolics than young leaves closer to the shoot apex. It has been well established that secondary metabolites usually accumulate at a definite developmental stage of the plant or plant organ (Wiermann, 1981).

Our results suggest that the strategy of *S. myrsinifolia* is to defend vulnerable shoots tips by the high concentration of salicylates, which will be diluted by tissue growth to the level observed in mature leaves. In mature leaves salicylates are stable compounds that do not need marked resynthesis. This is advantageous for plants, since salicylates are constitutive defenses that have to be kept at an effective level that offers protection against generalist herbivores, in which case a stable concentration with minimal turnover is the cheapest alternative.

Our prospective interest is to study the biosynthesis of more complicated salicylates such as salicortin, acetylsalicortin, and tremulacin and the turnover of salicylates in other *Salix* species. In addition, a study of the effects of AIP treatment on the growth of shoots and phenolic, nitrogen, and carbon content is in our interest.

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