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Seasonal Variation in the Hormone Content of Willow

I. CHANGES IN ABSCISIC ACID CONTENT AND CYTOKININ ACTIVITY IN THE XYLEM SAP[1]

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ABSTRACT

Changes in levels of abscisic acid (ABA) and cytokinin activity in the xylem sap of willow (*Salix viminalis*, L.) were followed throughout two growth cycles.

Growth in spring was preceded by decreasing levels of ABA and an increase in cytokinin activity. The onset of dormancy was associated with low levels of cytokinins and high contents of ABA. A second peak of ABA was found in July which was not related to the dry weight of the sap. The main cytokinin activity in the sap was due to a zeatin riboside-like compound.

The hormonal content of the xylem sap of woody plants has been the subject of several investigations. Davison (6) showed that the inhibitor levels in sap of *Salix fragilis* are higher in the dormant period than after bud break occurs. More recently, seasonal fluctuations in inhibitor levels have been reported in *Salix viminalis* with increasing amounts being found at the onset of dormancy (4). ABA was shown to participate in the inhibitory activity in the xylem sap of willow (11). This inhibitor was also identified in the sap from several woody species (7) and found to vary seasonally in xylem sap of peach, higher amounts being detected by GLC during the dormant period in the winter, with a small peak in midsummer (Davison and Young, in preparation). Cytokinins, gibberellins and auxins have also been found in sap of woody plants. Jones (10) demonstrated that xylem sap of apple tree promotes cell division in cultures of tobacco pith. Luckwill and Whyte (13) detected two main promoters in the sap of apple trees, one of them possessing cytokinin activity. Both appeared prior to bud swelling in March, attained their maximum concentrations at blossom time in May, and decreased thereafter, disappearing completely in October. These workers also mentioned experiments in which gibberellins and possibly auxins were found in bleeding sap of woody plants. Gibberellin and cytokinin activity were also found in the sap of *Acer pseudoplatanus* and *Betula pubescens* (16), *Vitis vinifera* (12, 18, 19), *Coffea arabica* (5), and *Populus robusta*. In poplar, no cytokinin activity was found during December and January, increasing amounts being found from 2 weeks prior to bud burst. Furthermore, the main activity detected was shown to be due to zeatin riboside-like component (9).

This work was carried out to determine whether or not ABA and cytokinin levels in the xylem sap of willow fluctuate seasonally, and whether the hormonal content of the sap can be correlated with the state of dormancy. ABA contents were measured by GLC and compared with the inhibitory activity detected by bioassays.

MATERIALS AND METHODS

Plant Material. As with many deciduous species of temperate regions, the growth of willow (*Salix viminalis* L.) follows a clearly defined pattern of seasonal changes. A period of dormancy lasting nearly 6 months is broken about mid-February when flower bud burst occurs. Flowering reaches its peak 1 month later, when leaf bud dormancy terminates and the phase of rapid shoot growth of summer and spring commences. As summer progresses, the rate of extension growth declines and ceases abruptly towards the end of August. The plants stop elongating long before temperature declines appreciably, the limiting factor for growth probably being the length of the day. One or two weeks after the cessation of extension growth all the growing points wither and abscise. Leaves start to yellow and fall from the lowermost part of the shoots, at more or less the same time as elongation growth stops. By early November all leaves have been shed, and the lateral buds remain in a dormant state until growth is resumed the following spring.

Stool shoots of willow were used in the course of the present work. Trees which are normally polled in late winter so as to produce only young shoots in the following season were used. Some were kept unpruned, however, to provide material for sap collections in the spring and early summer of the two seasons studied (1970-1972).

Observations of Growth. Extension growth was measured every 3 days on seven randomly selected shoots from midsummer until the internodes stopped elongating. Leaf fall was estimated by dividing the length of selected shoots into five equal segments and examining 10 of these shoots every week. A flower index was obtained by counting the number of opened flowers on 20 shoots at weekly intervals.

Collection of Xylem Sap. Sap was obtained from 4- to 15-month-old stems approximately 50 to 80 cm in length and 0.5 to 1 cm in diameter at the base, using the air displacement technique of Bollard (3). Shoots were harvested at random, at the middle of the day, defoliated or deblossomed, and immediately taken to the laboratory for extraction. The quantity of sap obtained by this method varied from 2.5 to 4 ml/shoot, or 1 ml of sap/35 g fresh weight of shoots, higher volumes being extractable during summer and autumn than during late winter and spring. The natural pH of the sap was 6 ± 0.77. Samplings were made at 2-week intervals during the 1st year, and at longer intervals in the 2nd. After extraction the sap was immediately filtered and frozen.

Determination of Total Solids. Total solids in the sap were measured by drying a minimum of six 2-ml aliquots in a desiccator to constant weight.

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Hormone Extraction for Bioassays. For each bioassay 20 ml of sap were used. Samples were acidified to pH 2.6 and extracted three times with equal volumes of diethyl ether. The aqueous layers were readjusted to pH 7, reduced to dryness *in vacuo*, at 30 C, and the residues were dissolved in a small volume of water for chromatography. The combined ether layers were dried, redissolved in ether-methanol (1:1, v/v) and chromatographed on paper.

Wheat coleoptile and soybean callus bioassays were performed with the ether-soluble acidic substances and the residual aqueous sap fractions, respectively. Acidic extracts were tested in groups of two or three. Several extracts (generally eight) were simultaneously tested in the soybean callus bioassay.

Paper Chromatography. Ether-soluble fractions, for wheat coleoptile assays, were streaked onto 10 cm wide, ethanol prewashed, Whatman No. 1 paper, and developed in isopropanol-ammonia-water (10:1:1, v/v/v). Aqueous fractions, for soybean assays, were loaded onto 22 cm wide Whatman No. 3MM paper and chromatographed in water-saturated secondary butanol. Descending chromatography was carried out in both cases, allowing the solvent front to reach a distance of 25 to 30 cm from the starting line.

Column Chromatography. Polyvinylpyrrolidone (GAF Ltd., Great Britain) was used in the purification of ABA for GLC analysis. Columns (28.5 × 1.5 cm) were packed and loaded with ammonium salts made from the acidic fractions of plant extracts. Elution was carried out with water, at a flow rate of 0.5 ml/min, collecting the fraction between 25 and 65 ml.

A Sephadex LH-20 (Pharmacia Fine Chemicals, Sweden) column was used for the resolution of sap cytokinins, as described by Hewett (9).

Thin Layer Chromatography. Plant extracts destined for quantitative analysis of ABA were dissolved in acetone-methanol (9:1, v/v) and strip loaded onto plates coated with a 0.25 mm layer of Kieselgel GF_{254} (Merck, Germany). Chromatoplates were developed in toluene-ethyl acetate-acetic acid (40:5:2, v/v/v). ABA was located by chromatographing a mixture of the authentic hormone on the same plate and examining under UV light (254 nm).

Gas-Liquid Chromatography. The purification of plant extracts and the quantitative analysis of ABA by GLC were carried out as described by Alvim (1). Briefly, radioactive ABA was added to the sap as internal standard. The sap was acidified to pH 2.6 and ABA was extracted in three equal volumes of diethyl ether. The ether layers were combined and shaken four times with quarter volumes of 5% (w/v) sodium bicarbonate. The bulked aqueous phases were acidified to pH 2.6, and the ether-soluble acids were re-extracted and dried. Ammonium salts were made and loaded onto the PVP column. The eluted volume known to contain ABA was reacidified to pH 2.6 and partitioned with three equal volumes of diethyl ether. The ether was evaporated, and the extract was redissolved in acetone and loaded onto thin layer plates. The ABA zone was eluted with acetone and evaporated to dryness. After purification the extracts were methylated with diazomethane (17), dissolved in an appropriate volume of acetone and injected into a Hewlett-Packard F&M chromatograph equipped with an electron capture detector. GLC was performed isothermally at 210 C, using columns packed with Epon 1001 as the stationary phase at a 2% loading on Aw-Dmcs Chromosorb W. The carrier gas employed was 95% argon, 5% methane, at a rate of 40 ml/min. Recovery in the purified extract was determined by radioactivity measurements on aliquots taken after the elution of the thin layer chromatograms.

Wheat Coleoptile Assay. The developed paper chromatograms were dried and cut into 10 R_F zones, each strip being placed in a vial (2.5 × 5 cm). Elution was accomplished by adding 1.2 ml of buffered 2% (w/v) sucrose solution (pH 5.3) to each of the vials and allowing them to revolve in a rotary wheel for about 1 hr. Wheat (var. Kolibri) seeds were germinated in the dark for 72 hr at 25 C. Coleoptiles, 18 to 22 mm in length, were selected and 1-cm sections were excised 3 mm below the apex. Ten sections were placed longitudinally in each vial, and their length was measured after 18 to 20 hr continuous rotation in a vertical orbit at 25 C in the dark. A straight analysis for variance was employed, using Duncan's multiple range test (8) to obtain the lowest significant difference of treatments from the control. Throughout this study the least significant difference was quoted at the 1% level.

Soybean Callus Assay. This test was carried out as described by Miller (15), three or four pieces of callus tissue being placed into flasks containing 25 ml of medium and one the 10 R_F zones. The cultures were maintained for 3 weeks at 26 C under constant fluorescent illumination, after which fresh weights were determined.

Liquid Scintillation Counting. Ten ml of a solution of toluene-Triton X (2:1, v/v) and 4 g/l PPO were added to each scintillation vial. Radioactivity was measured in a Beckman LS-200B liquid scintillation spectrometer, counting the samples to a standard error of 2%. Results were corrected for variations in counting efficiency by the external standard channels ratio method using a standard quench curve.

RESULTS

β-inhibitor activity in the acidic extracts was present between R_F 0.4 and 0.8, generally at 0.6 to 0.7. Main cytokinin activity was located at R_F 0.5 to 0.7, with a smaller peak at R_F 0.1 to 0.3.

Developmental changes occurring throughout the experimental period are diagrammatically depicted in Figure 1, together with total solids, daylength and seasonal fluctuations of cytokinin activity, ABA levels, and β inhibitor. Cytokinin activity increased abruptly at approximately the same time as floral bud burst, at least 6 weeks after an increase in total solids. A second

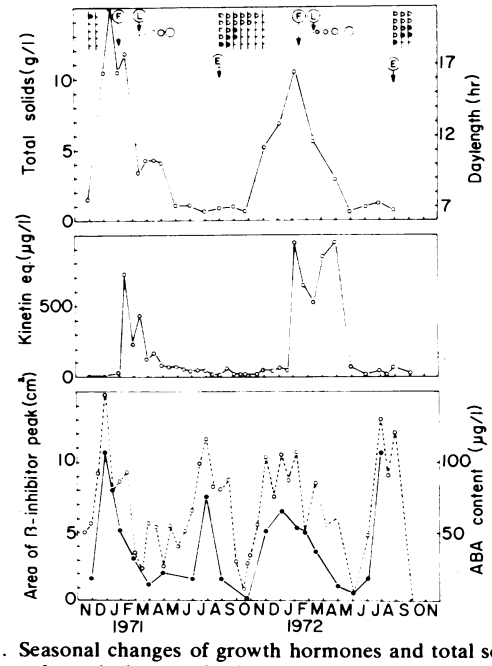

FIG. 1. Seasonal changes of growth hormones and total solids in the xylem sap of stool shoots of willow, daylength, and developmental changes occurring throughout the experimental period: Top: total solids, in g/l, daylength, in hours (.....), proportion of green (|○), yellow (|●) and abscised (|-) leaves per shoot, flower bud burst (F), leaf bud burst (L), leaf expansion (circles of different sizes), and cessation of extension growth (E); full blossom occurred concomitantly with leaf bud burst. Middle: cytokinin activity expressed as kinetin equivalents in µg/l, at R_F 0.5 to 0.8. Bottom: ABA contents in µg/l, and the β zone inhibitor (- - -) assessed as areas of histograms significantly different from control line at R_F 0.4 to 0.8.

peak appeared at the time when leaf buds burst, thereafter falling to, and remaining at low levels during the remainder of the summer. Partition chromatography of willow sap on Sephadex LH-20 indicated that the main activity had an elution volume similar to zeatin riboside. ABA concentration and inhibitory activity fluctuated throughout the year. Following leaf abscission, levels increased during winter, then decreased before growth commenced in the spring. A further increase occurred in July, before plants ceased extension growth. Minimum levels were obtained in October, after the shoot tips had abscised and only a few leaves remained on the plant. The autumn and summer variations were associated with parallel changes of the total solids in the sap, whereas the summer peak corresponded to the lowest value of sap dry weight. Except for the high levels detected during the summer months, therefore, the amount of inhibitors seemed to be a function of the state of dilution of the xylem sap.

DISCUSSION

Luckwill and Whyte (13) reported changes in cytokinin activity from apple xylem sap similar to those now found in willow. The results reported here also confirm the observations of Davison (6) that seasonal fluctuations in inhibitor content occur in xylem sap of willow, with high levels present during winter. These results are also in agreement with those found by Davison and Young (in preparation): they also detected the discrete summer peak, but of smaller magnitude than the one observed here. Failure to observe this peak in previous work at Aberystwyth (4) may have been due to less frequent sampling: the increase is transient, occurring shortly after the longest summer day, and inhibitor levels decrease to a minimum within a month.

Because of the method used for collecting the xylem sap, the concentrations of growth hormones measured represented those present in a given volume of stem at a given time. These may bear little relationship to the actual amounts reaching the apex and upper expanding leaves. This will depend on both the concentration and the rate of flow of the sap (which will be a function of the transpiration rate). Therefore in summer, when days are long and temperatures warm, with associated high transpiration rates, the actual amount of inhibitor reaching the apex will be considerably higher than the values found. On the other hand, concentrations obtained during winter, when the plants were leafless, depict more accurately the amounts eventually available to the buds, since flow rate in the xylem, if any, would be very low in the dormant period.

The marked seasonal changes observed in sap hormones were remarkably well correlated in time with growth rate and dormancy. It is therefore pertinent to discuss in detail some aspects of the seasonal patterns in ABA and cytokinin contents. The gradual accumulation of ABA following leaf fall paralleled changes in the weight of the sap, whilst cytokinin activity built up abruptly in the spring independently of the state of dilution of the sap. A possible interpretation of these changes, based on the fact that ABA is synthesized by roots of willow *in vitro* (1), is that both hormones are produced in the roots during the winter and accumulated in the xylem: ABA slowly and continuously, cytokinins suddenly and possibly requiring a long period of winter chilling. The maintenance of a dormant state during late autumn and winter could well be effected by ABA, in the presence of very small amounts of cytokinins. The considerable increases in cytokinins occurring prior to bud burst are clearly correlated with the growth pattern of the tree. On the other hand, a competition for available sap cytokinins between leaves and shoot growing tips, similar to that existing in the cacao tree (2), seems to occur in willow, in so far as activity decreased when leaves started to expand by the end of April.

It is interesting that bud burst occurred under the influence of dormancy inducing photoperiods. A possible explanation for this fact is that exposure to short days took place in the presence of declining amounts of ABA and high levels of cytokinins. An inverse situation happens when short days occur at the inception of the dormant period. It is also possible that the presence of mature leaves is necessary for the effectiveness of short days in inducing dormancy in willow, as suggested by Alvim (1). It is noteworthy and consistent with this hypothesis that ABA levels in the sap started to increase only after fully expanded leaves were formed. At this point in the annual growth cycle, sap ABA would be expected to originate in the leaves. Perhaps for this reason, sap ABA declined after the leaves started to yellow and abscise, reaching the lowest levels when only a few frost damaged leaves remained.

The advantages of using physical methods for detection and quantification of plant growth hormones have been stressed by Wareing and Saunders (21). A comparison of results obtained with the wheat coleoptile bioassay and GLC results suggests that ABA, if not the only inhibitor present in ether extracts of the xylem sap of willow, accounts for the largest part of its inhibitory activity. Although the general trends are satisfyingly similar, some discrepancies can be noted. In samples collected on 30/4 and 30/8, 1971 GLC indicated that there were 15 to 20 μg of ABA per liter of sap in each sample, while bioassays gave different inhibition responses, 2.6 and 8 cm^2 of histogram area, respectively. In other samples (30/11/70, 30/6/71, 30/4/72, and 3/7/72), approximately the same concentration of ABA was similar, more or less consistently, to an inhibition equivalent to 5 to 6 cm^2. To date, no physical methods for determining cytokinin levels from plant material have been reliably demonstrated, although the potential use of gas chromatography, mass spectrometry is well recognized (14, 20).

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