The Relationship between Carbohydrates and Flower Bud Hardiness among Three *Forsythia* Taxa

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Abstract. The accumulation of total soluble sugars (TSS) and starch and their relationship to flower bud hardiness were studied in three *Forsythia* taxa: *Forsythia* $\times$ *intermedia* 'Spectabilis', *Forsythia* $\times$ *intermedia* 'Lynwood', and *F. suspensa*. Taxon hardiness was based on the mean temperature at which low temperature exotherms (LTEs) occurred during thermal analysis. Ethanol-extracted soluble sugars were quantified with anthrone, and starch was enzymatically digested and quantified with Trinder reagent. Qualitative changes in sugar content were determined with high-performance liquid chromatography and co-chromatography of authentic standards. Quantitative and qualitative changes in sugar content, similar for the three taxa, were observed in conjunction with fluctuations in flower bud hardiness, although neither TSS nor starch were correlated with mean LTE temperature. TSS was higher in acclimated than nonacclimated buds. However, after deacclimation began, sugars continued to increase with mean LTE temperature. Buds lacked starch except for a brief period during deacclimation. Galactose, stachyose, raffinose, and an unidentified carbohydrate were positively correlated with hardiness ($P = 0.005, 0.001, 0.005, \text{ and } 0.001$, respectively).

The flower buds of many woody species undergo deep supercooling to avoid freeze injury during winter (Andrews and Proebsting, 1987; Ashworth, 1990; George and Burke, 1977; Graham and Mullin, 1976; Pierquet et al., 1977; Quamme, 1974, 1986; Rajashekar, 1989; Warmund and George, 1990). Among these species, which include several *Forsythia* taxa (Ashworth, 1990; data from parallel study), supercooling ability was confirmed by the occurrence of low-temperature freezing events or exotherms (LTEs) during a controlled freezing test. These LTEs coincided with the temperature at which lethal injury occurred within developing floral organs and therefore have been used as estimators of flower bud hardiness.

The development, maintenance, and subsequent dissolution of supercooling ability appear to be linked to the frequency of occurrences of daily subzero temperatures (Andrews and Proebsting, 1987; Quamme, 1983). However, freezing temperatures can be lethal when they occur in the form of a sudden, mid-winter freeze or a late spring frost subsequent to the loss of supercooling ability. Many physiological and biological changes are apparently involved in hardiness regulation including carbohydrate metabolism (Alden and Hermann, 1971; Kandler and Hopf, 1980), protein synthesis, gene expression (Guy, 1990), and alterations to membranes (Steponkus, 1984). Hardiness-related alterations in carbohydrate status have been studied in a wide range of plant species (Kandler and Hopf, 1980). Although a causal relationship has not been established, development of plant hardiness is typically associated with an increase in total soluble sugars (TSS) (Alden and Hermann 1971; Kandler and Hopf, 1980). In leaves and bark tissues of most plants, this increase arises concomitant with the hydrolysis of starch reserves. (Alberdi et al., 1989; Ashworth et al., 1993; Kandler and Hopf, 1980; Khalafalla

and Palzkill, 1990; O'Neill, 1983), although some exceptions have been reported (Alden and Hermann, 1971; Ristic and Ashworth, 1993). However, hardiness-related changes in the carbohydrates of flower buds have not been documented for species other than peach [*Prunus persica* (L.) Batsch] (Durner, 1989; Durner and Gianfagna, 1991; Lasheen and Chaplin, 1977; Layne and Ward, 1978). In those studies, the accumulation of TSS in flower buds differed from that observed in foliage and stem tissues in two respects: 1) acclimated flower buds were devoid of starch (Layne and Ward, 1978) and 2) the strong correlation between seasonal changes in hardiness and TSS that had been observed in leaves and stems was not observed in flower buds. In leaves and stems, levels of TSS typically declined with hardiness during deacclimation, whereas TSS in peach flower buds continue to increase through full bloom (Layne and Ward, 1978). However, some qualitative changes within TSS were highly correlated with bud hardiness (Lasheen and Chaplin, 1977) and were similar to those reported in leaves (Hinesley et al., 1992; Khallafala and Palzkill, 1990) and stems (Alden and Hermann, 1971; Ashworth et al., 1993; Kandler and Hopf, 1980). The sugars most often correlated with hardiness among all of these tissues were sucrose, raffinose, and stachyose, although concentrations varied among species and among plant tissues.

To determine whether hardiness-related changes in carbohydrates observed in peach flower buds are typical of reproductive buds in other species, we examined these relationships in the buds of three *Forsythia* taxa. The genus *Forsythia*, like *Prunus*, is comprised of numerous supercooling taxa (Ashworth, 1990; data from a parallel study). Although similar in hardiness, these taxa were differentially killed by a severe mid-winter freeze in 1991. Flower buds of *F. intermedia* 'Spectabilis' and *F. suspensa* survived to bloom the following spring while those of *F. intermedia* 'Lynwood' did not. To determine if carbohydrate composition of acclimated buds can differentiate among taxa with respect to hardiness limitations, this study was designed to answer the following questions: 1) What seasonal relationship, if any, exists among soluble sugars, starch, and hardiness in flower buds? 2) What individual carbohydrates comprise the soluble sugar fractions of the three taxa? and 3) Are any of these carbohydrates correlated with flower bud hardiness?

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Materials and Methods

Plant material. Terminal segments of the current season's growth of *F. suspensa* (Thunb.) Vahl., *Forsythia* $\times$ *intermedia* 'Spectabilis' (Koehne), and *Forsythia* $\times$ *intermedia* 'Lynwood' (G.E. Peterson) were collected from plantings on the Purdue campus in West Lafayette, Ind. Material was harvested during Winter 1992–93 at the following stages of hardiness: nonacclimated (9 Sept.), acclimated (11 Feb.–21 Mar.), and reacclimated (28 Mar.–9 Apr.). On each date, harvested materials were misted, sealed in plastic bags, and transported to the laboratory packed in ice. Upon arrival, the sealed bags were transferred to -4°C for a maximum of 2 h.

Thermal analysis. A modification of the thermal analysis procedure described by Ashworth (1990) was used to determine the depth of flower bud hardiness on each date. Leaves and all but one flower bud were removed from twelve 5-cm stem segments of each taxon. The remaining bud of each stem segment was taped to a 30-gauge (0.25-mm-diameter) copper-constantan thermocouple and placed in a stoppered test tube with 0.5 ml water and a chip of ice. The tubes were then cooled at $2^{\circ}\text{C}/\text{h}$ to -40°C in a circulating 50 ethanol :50 ethylene glycol (v/v) bath (components from Neslab Instruments, Newington, N.H.). Bud temperatures were recorded every 15 sec with a computer-based data-acquisition program. From resulting thermal profiles, the temperature preceding abrupt spikes in bud temperature were recorded as the LTE temperature. LTE temperatures were averaged for each taxon.

Daily minimum and maximum temperatures during the study were obtained from the Purdue Weather Service, located on campus in West Lafayette. The weather station was located ≈ 2 km from *Forsythia* plantings.

Carbohydrate analysis. In preparation for carbohydrate analysis, flower buds were excised from stems, quench frozen in liquid nitrogen, and freeze-dried. Three replicates of 50 lyophilized buds of each taxon on each date were weighed, ground in a Wiley mill to pass through a 60-mesh screen, and stored at -80°C . Fifty-milligram aliquants of each milled replicate, along with an equal weight of polyvinylpyrrolidone (Sigma Chemical Co., St. Louis) and 250 μl of 10 mM lactose (internal standard), were extracted with 1 ml 80% (v/v) ethanol for 15 min and centrifuged at $4000\times g$ for 5 min, and the supernatant was decanted. The pellet was extracted two additional times. Supernatants were pooled and brought to a final volume of 10 ml with 80% ethanol. Total sugars were then measured as glucose equivalents in triplicate replicates of each sample with anthrone (Van Handel, 1968). Portions of extracts were stored at -80°C for later quantification of individual sugars.

Ethanol-extracted pellets were dried overnight at 80°C and then resuspended in a 50 mM sodium acetate buffer (pH = 5.2). Starch in the pellets was then digested to glucose by incubating the pellets for 12 h at 57°C with 400 units/ml α -amylase and 2 units/ml amyloglucosidase in 50 mM sodium acetate buffer. After centrifugation at $4,000\times g$ for 5 min., triplicate aliquots of 200 μl of supernatant from each sample was brought to a final volume of 1 ml and was quantified with glucose Trinder reagent (Sigma Chemical Co.).

Identification of individual sugars. Qualitative analysis of ethanol-extracted sugars was accomplished by methods described by Ashworth et al. (1993). Briefly, ethanol was evaporated from 0.5 ml extract of each sample. Sugars were redissolved in 1 ml deionized water and filtered through a 0.45- μm nylon membrane. Extracts were separated into their component peaks by injecting 25

Table 1. Seasonal changes in flower bud hardiness and carbohydrate components of *Forsythia* flower buds. Each LTE value represents the mean of 10 replications and each sugar value represents the average of 3 replications.

Sample date	Taxon	Mean LTE ($^{\circ}\text{C}$)	Sugar (mg·gm $^{-1}$ dry wt)				
			Galactose	Glucose	Fructose	Sucrose	Melibiose
9 Sept.	Lynwood	-5.0	0.62	63.99	14.71	52.00	0.00
	Spectabilis	-5.0	0.35	54.89	6.51	40.44	0.00
	<i>F. suspensa</i>	-5.0	0.31	52.60	8.07	51.25	0.00
11 Feb.	Lynwood	-20.4	6.07	74.35	30.84	2.67	6.09
	Spectabilis	-20.6	6.20	70.56	30.48	5.14	5.36
	<i>F. suspensa</i>	-20.7	4.88	77.07	27.17	3.47	4.10
1 Mar.	Lynwood	-19.8	7.22	73.23	38.85	2.38	7.17
	Spectabilis	-20.7	6.84	62.81	39.15	1.06	6.67
	<i>F. suspensa</i>	-20.4	6.93	72.15	39.35	1.39	6.66
14 Mar.	Lynwood	-20.1	5.47	71.87	30.34	12.69	6.29
	Spectabilis	-21.2	6.67	70.05	31.96	1.54	7.18
	<i>F. suspensa</i>	-20.4	4.83	62.40	31.99	19.37	5.01
21 Mar.	Lynwood	-17.4	6.53	71.43	21.21	0.00	6.61
	Spectabilis	-17.9	6.45	70.81	22.90	0.21	6.84
	<i>F. suspensa</i>	-18.5	5.62	71.39	23.10	0.27	5.39
28 Mar.	Lynwood	-15.9	5.54	72.46	17.82	0.00	5.12
	Spectabilis	-14.7	5.34	72.23	18.28	0.00	5.15
	<i>F. suspensa</i>	-15.6	4.77	74.09	17.65	0.00	4.09
4 Apr.	Lynwood	-11.7	4.59	73.90	26.44	0.00	5.19
	Spectabilis	-12.4	3.31	76.17	44.61	0.00	1.55
	<i>F. suspensa</i>	-10.4	4.68	75.47	23.69	0.00	4.26
9 Apr.	Lynwood	-5.7	3.65	68.18	46.31	0.00	2.08
	Spectabilis	-5.5	5.37	76.53	27.42	0.00	5.01
	<i>F. suspensa</i>	-6.6	4.28	76.35	65.46	2.08	1.92

μl each sample into a high-performance liquid chromatography (Dionex, Sunnyvale, Calif.) equipped with a pulsed amperometric detector and a CarboPac PA1 pellicular resin column (4 × 250 mm). Peaks were identified by comparison with retention times of authentic standards of arabinose, galactose, glucose, fructose, sucrose, melibiose, lactose, raffinose, stachyose, and maltose.

Results

Environmental temperature. No natural freezes occurred before the first collection date. For subsequent collection dates, minimum temperatures below 0°C had occurred. January's daily minimum temperatures ranged from -11 to 2°C and were below 0°C

Fig. 1. Seasonal fluctuation of carbohydrate content and cold hardiness of three *Forsythia* taxa; Carbohydrate values are the average of three replicate assays and mean LTEs are averages of 12 replicates of buds collected during Winter 1992. Error bars indicate the standard deviations. Taxa are *Forsythia* 'Lynwood' (a), *Forsythia* 'Spectabilis' (b) and *F. suspensa* (c). Total soluble sugars were not correlated with starch levels [$r = 0.084$ (a), 0.401 (b), 0.367 (c)] or with hardiness [$r = 0.265$ (a), 0.028 (b), 0.408 (c)]. Starch was not correlated with hardiness either [$r = 0.249$ (a), 0.161 (b), 0.303 (c)].

on 23 days. Daily minimum temperatures for February were much colder, ranging from -22 to 1C with a total of 26 days below 0C. A warming trend began in March with daily low temperatures ranging from -11 to 12C and only 11 days below 0C. By April, low temperatures ranged from -4 to 14C, with only 5 days below 0C. The winter was punctuated with 2 weeks 'of abnormally warm temperatures of 11 to 16C beginning on 1 Feb.

Flower bud hardiness. Flower bud hardiness, similar among the taxa throughout the season, followed the same trends observed in minimum daily temperatures. The most cold-sensitive buds in the study were collected on 9 Sept., before the first autumn freeze. By 11 Feb., buds had acclimated to about -20.5C and remained at that level of hardiness through 14 Mar. Over the next weeks, buds reacclimated with mean LTEs for all three taxa increasing at the rate of 2-4C per week. On the last collection day, 9 Apr., buds were no longer hardy, as was demonstrated by mean LTEs of -5.7, -5.5, and -6.6C for 'Lynwood', 'Spectabilis', and *F. suspensa* (Table 1).

Carbohydrates. Seasonal soluble sugar content was similar among the three taxa. TSS increased from 10%, 11%, and 12% of bud dry weight for 'Lynwood', 'Spectabilis', and *F. suspensa*, respectively (Fig. 1), on 9 Sept. to $\approx$ 12% to 13% of dry weight by 11 Feb., where they remained through 14 Mar. Soluble sugars initially declined during deacclimation, but subsequently increased. At the last sampling, TSS had risen to $\approx$ 16% of bud dry weight, the highest levels observed during the study.

The amount of starch in *Forsythia* flower buds was negligible at the first four sampling dates (Fig. 1). Beginning in mid-March, starch levels rose in all three taxa to a maximum of $\approx$ 2% and 4% ('Spectabilis') bud dry weight. However, by the last sampling date, starch levels had returned to undetectable amounts.

The composition of extracted sugars of *Forsythia* flower buds, a complex mixture of >20 carbohydrates, changed over the course of the study, although compositions were similar among taxa (Table 1). Concentration of sorbitol, myo-inositol, arabinose, galactose, glucose, fructose, sucrose, melibiose, raffinose, and stachyose in flower bud extracts were determined via standard curves of individual components. One carbohydrate was tentatively identified as lactose in preliminary trials. However, subsequent analysis of a lactose-spiked sample revealed the unknown component to be a different carbohydrate with elution times similar to that of lactose. Arabinose, sorbitol, and myo-inositol

were relatively stable throughout the season and therefore were precluded from subsequent analyses.

Correlation coefficients calculated for the relationships between individual sugars and TSS, starch, and hardness revealed that glucose and fructose, which were the predominant sugars throughout the season, were linearly correlated with TSS ($P > 0.005$) (Fig. 1, Table 2). Although glucose was correlated with hardness also ($F' = 0.05$), coefficients of the following sugars, listed in order of decreasing concentration, were more significant: galactose, stachyose, a second unidentified carbohydrate, and raffinose ($P = 0.005, 0.001, 0.001$, and 0.005 , respectively). The concentration of the second unidentified carbohydrate, with elution times intermediate to those of melibiose and raffinose, was based on the molecular weight of the trisaccharide raffinose and was present in amounts comparable to stachyose (Fig. 2). Raffinose, stachyose, and this second unknown were present only in acclimated and reacclimating buds. As a molar fraction of identified carbohydrates, their concentrations declined steadily throughout dormancy from a combined maximum of 5% in February to 0% by 4 Apr. Differences in mean sugar concentrations of raffinose and stachyose were not significant among taxa. However, on 11 Feb., concentrations of the unknown were highest in buds of 'Lynwood' and 'Spectabilis' and on 1 Mar. in 'Spectabilis'. Sucrose was more abundant in nonacclimated buds than in any other buds, and, like melibiose, was not correlated with hardness.

Discussion

Flower bud hardiness is affected by many environmental factors, not the least of which is temperature (Andrews and Proebsting, 1987; Graham and Mullin, 1976; Quamme, 1983). *Forsythia* flower buds were no exception. Mean LTE temperatures in all three taxa followed trends in daily minimum temperatures. Similar to bud hardiness in sweet cherry (Andrews and Proebsting, 1987), the depth of *Forsythia* hardiness appeared to be unaffected by a brief period of unseasonably warm temperatures that occurred in February.

The accumulation of soluble sugars during cold acclimation has been observed in a diverse range of plant species (Alden and Hermann, 1971; Kandler and Hopf, 1980). Like peach buds (Layne and Ward, 1978), TSS was higher in acclimated *Forsythia* buds than in nonacclimated ones (Fig. 1). However, unlike TSS in

Table 2. Matrix of correlation coefficients for hardness, total carbohydrates, and individual sugars.

<i>Forsythia</i>		Pearson's correlation coefficient (<i>r</i>)								
taxon	TSS ^a	TNS ^b	Galactose	Glucose	Fructose	Sucrose	Melibiose	Unknown	Raffinose	Stachyose
Lynwood										
MLTE ^c	0.29	0.48	-0.82***	-0.67*	0.07	0.19	-0.48	-0.83***	-0.74***	-0.84***
TSS		0.08	0.37	0.87***	0.92***	-0.55	0.06	0.16	0.25	0.16
TNS			0.04	0.27	-0.27	0.18	-0.01	-0.38	-0.59	-0.47
Spectabilis										
MLTE	-0.10	0.25	-0.72**	-0.71**	-0.03	-0.28	-0.53	-0.84***	-0.68*	-0.83***
TSS		0.40	0.52	0.86***	0.74**	-0.58	-0.27	-0.04	0.01	0.08
TNS			-0.01	0.72	0.43	0.30	0.26	0.52	-0.45	0.52
<i>F. suspensa</i>										
MLTE	0.47	0.41	-0.76***	-0.34	0.30	0.30	-0.49	-0.92***	-0.71**	-0.88***
TSS		0.37	0.55	0.95***	0.96***	-0.23	-0.08	-0.33	-0.24	0.38
TNS			0.22	0.51	0.20	-0.44	-0.31	-0.53	-0.55	-0.67*

^aTotal soluble sugars.

^bTotal nonsoluble sugars.

^cMean LTE temperature.

*, **, ***Significant at $P = 0.05, 0.01$, or 0.005 , respectively.

Fig. 2. Seasonal course of individual sugars in buds of three *Forsythia* taxa. Sugar values are the average of three replicates with error bars indicating standard deviations. Taxa are *Forsythia* 'Lynwood' (a), *Forsythia* 'Spectabilis' (b) and *F. suspensa* (c).

leaves and stems of other genera (Alberdi et al, 1989; Alden and Hermann, 1971; Ashworth et al., 1993; Kandler and Hopf, 1980; Khalafalla and Palzkill, 1990; Lasheen and Chaplin, 1977; O'Neill, 1983), the seasonal changes in TSS among *Forsythia* taxa buds were not correlated with fluctuations in flower bud hardness (Table 2). This and the lack of starch in nonacclimated and acclimated buds is also typical of peach flower buds (Layne and

Ward, 1978). Lasheen and Chaplin (1977) concluded that the increase in soluble sugars accompanying acclimation in flower buds had been translocated from other parts of the plant. This also appeared to be the case in acclimated *Forsythia* buds. However, unlike peach (Layne and Ward, 1978), *Forsythia* buds were not devoid of starch the whole season (Fig. 1). Simultaneous to a decline in TSS and the onset of deacclimation, carbon partitioning

apparently switched from soluble sugars to starch. During a subsequent resumption in TSS accumulation, starch was depleted, giving rise to an increase in the molar fraction of monosaccharides as flowers reached full bloom. The physiological significance of this shift in metabolism is unknown.

Glucose and fructose were just 2 of >20 carbohydrates that comprised soluble sugars in *Forsythia* taxa. One as yet unidentified sugar appeared to co-chromatograph with lactose. In view of earlier reports indicating the presence of lactose in the pollen of two of the *Forsythia* taxa used in the present study (Avigad, 1982; Kuhn and Low, 1949), soluble sugar extracts before and after the addition of lactose as an internal standard were qualitatively compared. The lack of naturally occurring lactose was confirmed by the presence of an additional peak in lactose-spiked samples, although elution times of lactose and this unknown were often <0.5 min apart. Although the presence of lactose had been previously refuted (Toba et al., 1991), the presence of a carbohydrate with chromatographic properties comparable to those of lactose was not mentioned. As the results of our study indicate, this unidentified sugar may have been mistakenly identified as lactose in earlier literature (Avigad, 1982; Kuhn and Low, 1949).

The gain and subsequent loss of cold hardiness in overwintering plant tissues have often been linked with similar changes in the concentrations of sucrose, sorbitol, raffinose, and stachyose (Alden and Hermann, 1971; Ashworth et al., 1993; Hinesley et al., 1992; Kandler and Hopf, 1980; Lasheen and Chaplin, 1977). The relationship between the first two sugars and *Forsythia* flower bud hardiness differed from that observed in peach flower buds (Lasheen and Chaplin, 1977). In the present study, sucrose was lower in nonacclimated than in acclimated buds, and sorbitol levels remained stable during the winter (Table 2; sorbitol data not presented). However, flower bud hardiness was highly correlated not only with raffinose and stachyose, as in peach flower buds (Lasheen and Chaplin, 1977), but also with a second unidentified carbohydrate. Although attempts were made to identify this carbohydrate by comparing its elution times with those of trehalose, mannose, xylose, maltose, and verbascose, it remains unidentified.

The roles that raffinose and stachyose play in plant cold hardiness have not been established, although circumstantial evidence suggests that a physiological role may exist. In *Pinus excelsa* seedlings, for example, the induction of frost resistance at 20C by exposure to short-day photoperiods also resulted in the accumulation of raffinose, whereas long day treatments at the same temperature resulted in neither frost resistance nor raffinose accumulation (Kandler and Hopf, 1980). The presence of raffinose and stachyose in conjunction with sucrose appears necessary to confer desiccation tolerance to membranes (Hoekstra et al., 1992; Koster and Leopold, 1988; Koster, 1991). These sugars are thought to replace lost water in hydrophilic interactions, reduce toxic concentrations of chaotropic solutes, or provide structural support through glass formation. And since plant tissues undergo a freeze-induced drought as cellular water migrates toward growing extracellular ice crystals, this may be a potential role for raffinose and stachyose in freeze-resistant flower buds as well. In this capacity, even concentrations as small as those observed in acclimated *Forsythia* buds have been demonstrated to be effective (Koster, 1991).

Transport of photosynthetic assimilates from source tissues to sinks in other parts of the plant is achieved via transport of carbohydrates, which are often family-specific (Durner, 1989; Durner and Gianfagna, 1991; Madore, 1990; Mitchell and Madore, 1992). For example, genera of the Rosaceae family translocate carbohydrates in the form of sorbitol (Durner, 1989; Durner and Gianfagna, 1991), while stachyose is the primary transport carbo-

hydrate for members of the Lamiaceae family (Madore, 1990; Mitchell and Madore, 1992). Although the sugar composition of *Forsythia*, a member of the Oleaceae, was unknown before the current study, labeling studies had been conducted with other members of the same family (Kandler and Hopf, 1980). Those studies indicated that sugars in the leaves were transported to stems and roots as raffinose (Kandler and Hopf, 1980). Whether raffinose serves as a transport carbohydrate in *Forsythia* is not known.

What do the results of this study mean with respect to our original hypothesis that sugar composition could be used to predict hardiness limits of *Forsythia* taxa? Unfortunately, the lack of significant differences in cold hardiness among the taxa studied precluded a thorough test of the hypothesis. However, correlations between the levels of raffinose, stachyose, and an unknown carbohydrate with cold hardiness indicates that one or a combination of the three might serve as indicators of flower bud hardiness. Even more promising was the observation that the taxon with the largest concentration of this unknown component had the lowest mean LTE temperature, although differences in hardiness were not significant among the taxa. Similar studies conducted on *Forsythia* taxa with a broader range of flower bud hardiness than those used in the current study would indicate whether this sugar might serve as a selectable marker for cold tolerance.

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