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Sugar and Proline Accumulation in Grapefruit Flavedo and Leaves during Cold Hardening of Young Trees[1]

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Abstract. **Four-year-old 'Marsh' grapefruit (*Citrus paradisi* Macf.) trees on trifoliate orange [*Poncirus trifoliata* (L.) Raf.] rootstock were temperature acclimated to 5°C in controlled environment facilities with approximately 400 μeinsteins $m^{-2}s^{-1}$ PAR. Total soluble carbohydrates and proline increased in both leaves and fruit flavedo as temperatures were progressively decreased. Maximum accumulation of carbohydrates occurred in leaves and flavedo at 10° ambient air. Both sucrose and reducing sugars increased in leaves at all acclimating temperatures, but only reducing sugars increased in the flavedo at temperatures below 15°. The concentration of proline was the greatest in the leaves and flavedo at 5°. Both total soluble carbohydrates and proline decreased during temperature deacclimation at 25°.**

Accumulation of sugars and proline in citrus tissues is associated not only with cold hardening of trees (14, 15, 16), but also with the midseason resistance of grapefruit to chilling injury (6, 7, 8). Accumulation of reducing sugars and proline in grapefruit flavedo (colored portion of rind) seemingly would follow the general cold hardening of the tree, since grapefruit as well as several other citrus forms are often harvested in mid- or late-season and are exposed to the same ambient winter air temperatures as the tree. Temperature-induced cold hardening of citrus trees may simultaneously increase the resistance of the fruit to chilling injury, a major problem in the storage of grapefruit (2). However, previous observations in central Florida did not show the concentration of reducing sugars in grapefruit flavedo was significantly correlated with average low temperatures in the grove (8). Lack of a significant correlation may have been due to the wide flucuation in weekly high and low temperatures common in central Florida during winter months or to some other environmenal factor.

Objectives in this study were (a) to evaluate shifts in reducing sugars and proline in grapefruit flavedo on trees under controlled temperature regimes, and (b) to relate changes in sugar and proline concentrations in the flavedo to changes in sugar and proline concentrations in the leaves during cold hardening. Shifts in reducing sugars, sucrose, and proline in flavedo of detached fruit stored at 5°C were made for comparison.

Materials and Methods

Trees. 'Marsh' grapefruit budded on trifoliate orange were used in all test trials. Trees growing outdoors in a soil mix of 1 sand:1 sphagnum peatmoss:1 vermiculite (v/v/v) in 38-liter containers were obtained from a commercial citrus nursery. Trees were 4 years old, about 110 cm tall with 9-cm trunk diameter 5 cm above the bud union and averaged 8- to 11-cm-diameter fruit per tree. Two-tree replicates were used in each temperature-acclimation regime.

Temperature acclimation regimes. Grapefruit trees were temperature acclimated during November and December of 1980 in controlled environment walk-in rooms utilizing Cool White fluorescent and incandescent lighting to supply approximately 400 μE $m^{-2}s^{-1}$ (PAR) during 12-hr photoperiods (12). Although this light intensity is only about ⅕ full sunlight, it is sufficient to harden citrus (12). Temperatures were maintained within ∓0.5°C. Automatic steam injection maintained relative humidity at 60 ∓ 5%. Trees were watered daily to maintain the soil at near field capacity. Trees were divided into 2 sets of 2 trees each after 7 continuous days at a constant 25°, and acclimation temperature regimes were imposed. One set of trees was maintained at 25°, while the other set was progressively cold hardened by successive 7-day exposures to 20, 15, 10, and 5°, respectively. The temperature was increased after 1 week at 5° to 25° for an additional 2 weeks for deacclimation.

Freeze tolerance. Freeze tolerance tests were made using detached leaves which were arbitrarily selected from trees after each respective temperature acclimation period. Leaves were misted with deionized water to prevent supercooling of leaves, and were placed side by side in 0.013-mm polyethylene wraps. Wraps containing a row of 10 leaves per tree per temperature regime were suspended in a freeze room. Freezing tests were conducted in the dark at 50 ∓ 5% relative humidity. Tests began with 2 hr of 4.4°C, followed by a 1.1°/hr decrease to −6.7° for 4 hr, and ended with a return to 4.4° at a rate of 1.1°/hr. Frozen leaves were immediately transferred to a continuous mist in a greenhouse and

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maintained for 48 hr. Leaves were then observed for permanent water-soaked areas (a marker for lethal freeze injury) which were measured in cm^2 and converted to percentage of total leaf area.

Tissue analyses. Tissue analyses were made on individual leaves and fruit. Two leaves and either 1 or 2 fruit were sampled from each tree at the end of each temperature acclimation period. Samples were kept on ice until extractions were begun. Extraction and analyses of total soluble carbohydrates and reducing sugars were carried out as previously described (7). In addition, samples of the resin-treated ethanol extract were silated with Tri-Sil[2] (Pierce Chem. Co., P. O. Box 117, Rockford, IL 61105) and sugars were determined by gas chromatography (5). Proline was determined on ethanol extracts before resin treatment by the procedure of Ting and Rouseff (10). Starch was extracted from the ethanol-insoluble residue of leaf tissue by the perchloric acid methods of Clegg (1). Analysis was by the anthrone method.

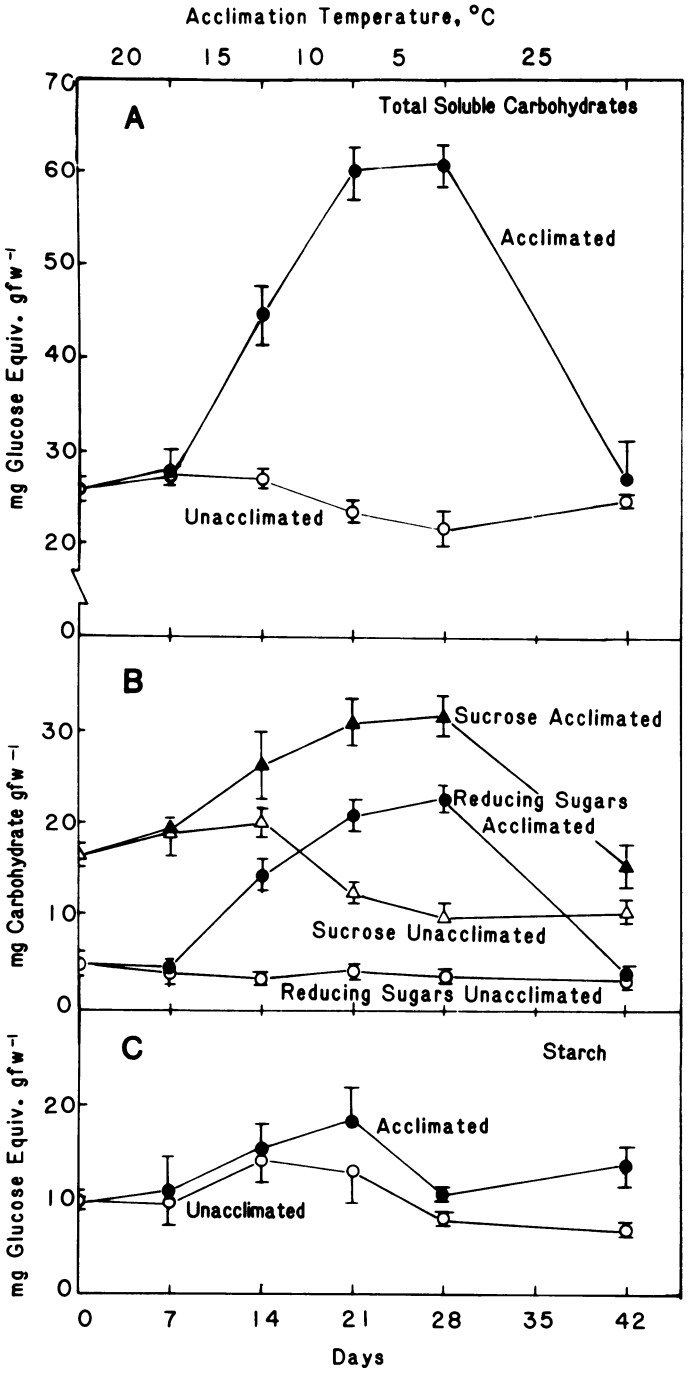

Fig. 1. Carbohydrate levels in grapefruit leaves acclimated by successive 1-week exposures to temperatures decreased progressively each week by 5°C compared to leaves of trees maintained at 25° through the entire experimental period. A. Total Soluble Carbohydrates. B. Sucrose and Reducing Sugars. C. Starch. (Bars represent SE.)

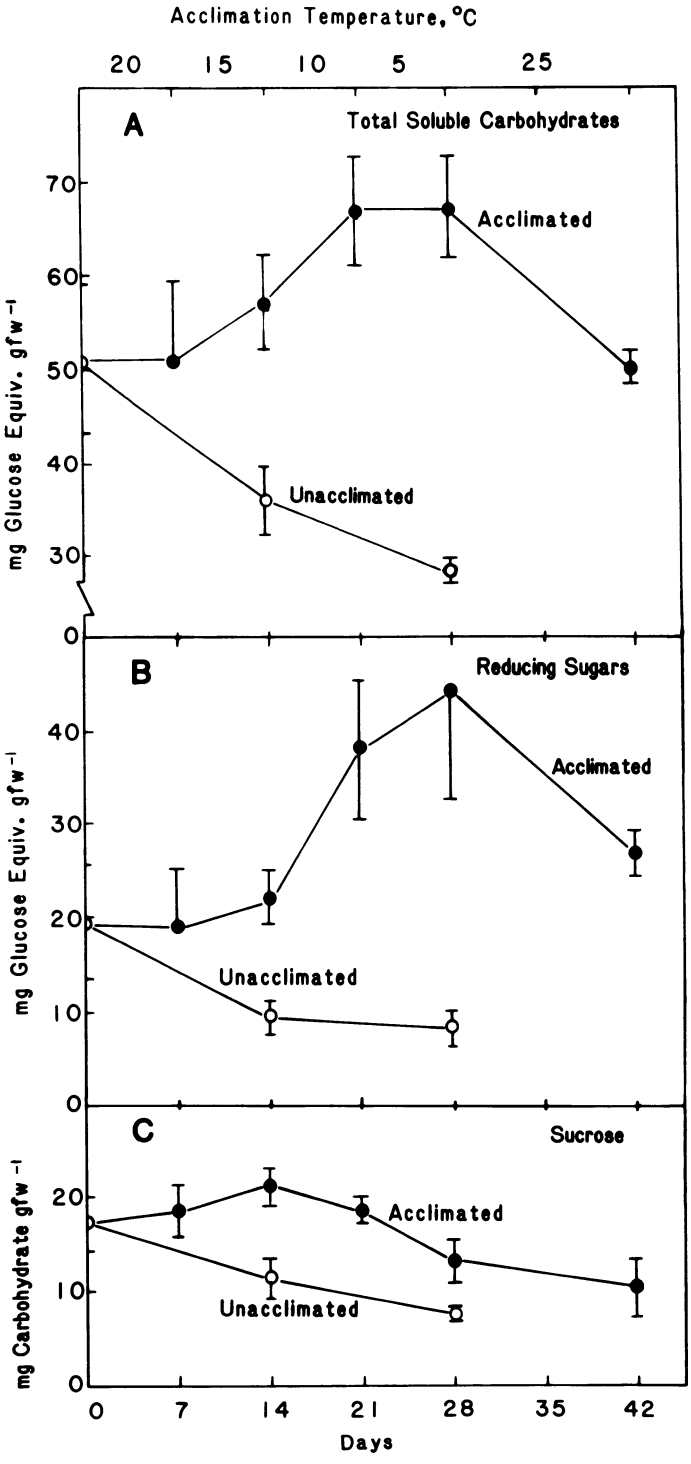

Fig. 2. Carbohydrate levels in flavedo tissue of fruit from same trees as in Fig. 1. A. Total Soluble Carbohydrates. B. Reducing Sugars. C. Sucrose (Bars represent SE.)

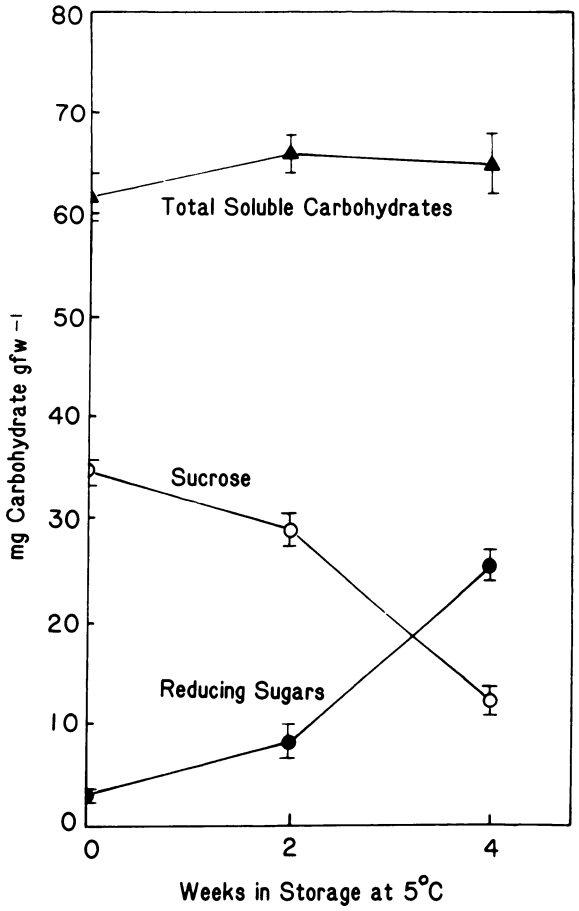

Fig. 3. Changes in total soluble carbohydrates, sucrose, and reducing sugar content in grapefruit flavedo during storage at 5°C. (Each point is the mean of 6 samples from 2 separate experiments. Bars represent the SE.)

Detached fruit were stored at 5°C and flavedo samples were analyzed for total soluble carbohydrates, reducing sugars, sucrose and proline initially and after 2 and 4 weeks of storage.

Results and Discussion

Carbohydrates increased in grapefruit leaves during cold acclimation of the trees. (Fig. 1). Total soluble carbohydrates began accumulating in leaves at 15°C with maximum accumulation at 10°. Both sucrose and reducing sugar levels increased in leaves during cold acclimation. Starch content also increased as temperatures were decreased to 10°. In contrast to soluble carbohydrate levels, starch content declined at 5°. Total soluble carbohydrates, reducing sugars and sucrose decreased to preacclimation levels during the 2-week deacclimation at 25° (Fig. 1). Total soluble carbohydrate concentration did not change in the leaves of trees maintained at a constant 25° (Fig. 1). Sucrose content declined slightly whereas reducing sugar concentration was low initially and did not change during the experimental period.

The source of soluble carbohydrates accumulating in grapefruit leaves during cold acclimation is not known. It is unlikely the sugars resulted from starch hydrolysis, since starch hydrolysis, i.e. a decrease in starch concentration, was not indicated until temperatures were decreased to 5°C. Yelenosky and Guy (15) favor photosynthesis over starch hydrolysis in maintaining sugar increases in 'Valencia' orange leaves at low but nonfreezing temperatures. Starch concentration decreased in 'Valencia' orange leaves between 5 and 0°. Accumulation of soluble carbohydrates in grapefruit leaves during acclimation could result from reduced translocation at low temperatures.

Soluble carbohydrates also accumulated in grapefruit flavedo during cold acclimation of the trees (Fig. 2). The pattern of accumulation was similar to that in leaves. A higher concentration of soluble carbohydrates was present in flavedo than in leaves initially and the increase in concentration in flavedo was less than that in leaves during cold acclimation. In contrast to leaves where sucrose and reducing sugar increased similarly during cold acclimation, only reducing sugar content increased in flavedo tissue at temperatures below 15°C while sucrose levels declined (Fig. 2). Total soluble carbohydrates, sucrose and reducing sugar content declined to the approximate preacclimation levels during the 2-week deacclimation at 25°. Total soluble carbohydrates, reducing sugar and sucrose content decreased in the fruit flavedo of trees maintained at a constant 25°.

Indirect evidence suggests leaves are the source of soluble carbohydrates accumulating in the flavedo during cold acclimation, since neither starch nor starch-degrading enzymes where detectable in flavedo tissue (8). Citrus fruits are strong sinks for photosynthates (3) and their presence may direct translocation even at low but nonfreezing temperatures. In addition, the concentration of total soluble carbohydrates did not change significantly in detached fruit maintained at 5°C, but reducing sugar concentration increased and sucrose concentration decreased (Fig. 3). Therefore, the increase in reducing sugar concentration in the flavedo of fruit attached to the tree during periods of low temperature may be partly due to the hydrolysis of sucrose.

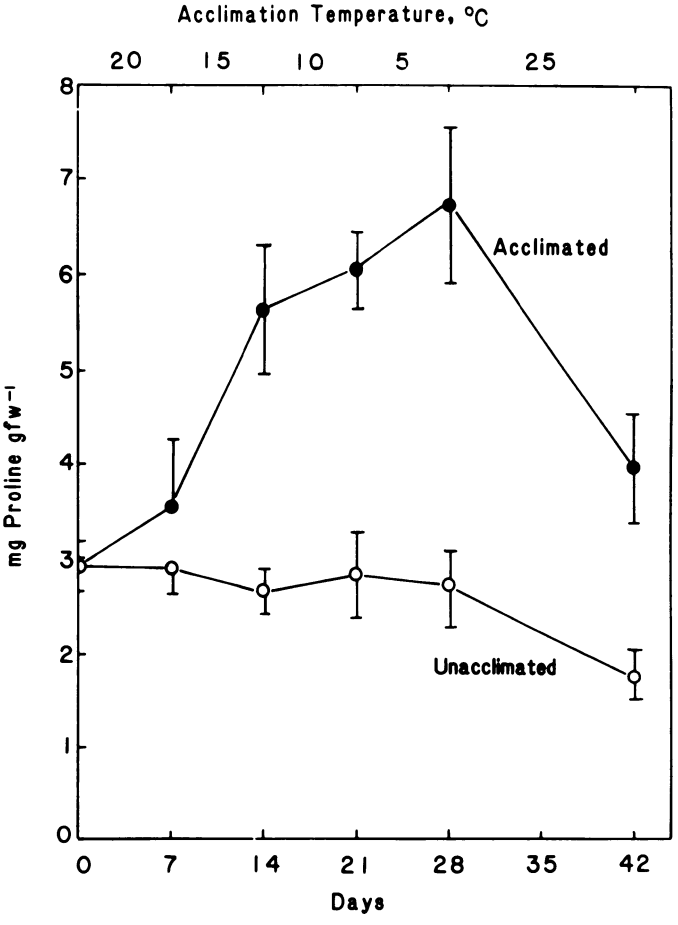

Fig. 4. Proline levels in leaves of same grapefruit trees as in Fig. 1. (Bars represent SE.)

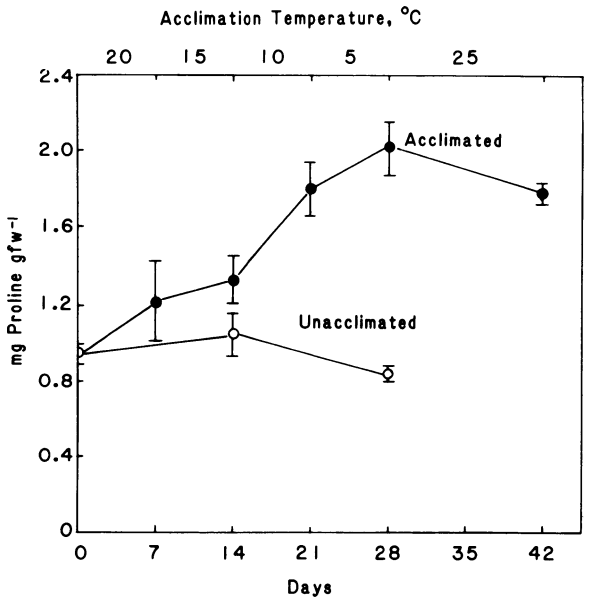

Fig. 5. Proline levels in flavedo tissue of fruit from same trees as in Fig. 1. (Bars represent SE.)

Proline increased in grapefruit leaves during cold acclimation with the largest increase at 15°C (Fig. 4). The proline content did not change in leaves of trees maintained at 25° and declined in the leaves of acclimated trees during the 2-week deacclimation at 25°.

Proline also increased in fruit flavedo during cold acclimation (Fig. 5). In contrast to leaves, the largest increase was at 10°C. In addition, proline concentration in the flavedo was always lower (approximately 3-fold) than in leaves at comparable temperatures. Whether proline was synthesized in the flavedo or translocated from the leaves was not determined. However, proline concentration did not change in detached fruit stored at 5° for 4 weeks.

An increase in soluble carbohydrates and proline has been used to characterize increased cold hardiness of citrus seedlings (14, 15). Leaves of trees exposed to progressively colder temperatures in this study also had increased resistance to freezing injury (Table 1). Unfortunately, we were unable to evaluate resistance of the fruit to chilling injury because of the inadequate number of fruit available relative to available space in controlled environment rooms. However, previous studies have shown significant correlations between the accumulation of reducing sugars and proline and increased resistance of the fruit to chilling injury (6, 7, 8). The role of sugars and proline in cold hardiness and resistance to chilling injury is not known. Accumulation of sugars preceded maximum cold hardiness of 'Valencia' orange seedlings and maximum resistance of grapefruit flavedo to chilling injury by about 4 weeks (7, 8, 15). Steponkus (9) presents evidence that cold acclimation of *Hedera helix* is a 2-phase process, the first phase being the accumulation of sugars. The second phase is thought to involve the production of proteins which, due to an altered composition or configuration, have a greater capacity to bind sugars and are thus protected from denaturation at freezing temperatures.

Table 1. Cold hardiness of leaves of 'Marsh' grapefruit trees exposed to progressively colder temperatures (5°C/week) and followed by 2 weeks deacclimation at 25°C.

Successive weekly temp. treatment (°C)	Damage (%)[z]	
	Acclimated	Unacclimated[y]
25	100	100
20	—	—
15	51 ± 5[x]	100
10	—	—
5	20 ± 5	100
25	—	—
25	99 ± 1	100

[z]Hardiness is expressed as the percentage of permanent water soaking in the leaves 48 hr after leaves were exposed to -6.7°C for 4 hr with freezing and thawing rates of 1.1°/hr.
[y]Trees maintained at 25°C.
[x]Mean ± SE (n=10).

Acclimation or hardening has generally been regarded as a freezing survival mechanism of vegetative tissues in deciduous fruit culture (4) and citriculture (13). Leaves and fruits of deciduous trees abscise and/or are harvested before the onset of winter temperatures. Consequently, acclimation of leaves and fruit is not often studied simultaneously. However, grapefruit is a subtropical evergreen and the fruit generally is on the tree well into the winter season and often until the following spring. Therefore, fruit and leaves are frequently exposed to the same winter temperature conditions. It has been suggested that roots and stems will harden to the same degree when acclimated at similar temperatures (11). Hence, the cold hardening regime may be a more important determinant of the degree of hardening than the particular organ.

This is the first study we are aware of indicating fruit acquire characteristics of hardiness concomitant with vegetative tissues during cold acclimation. Since the increases in soluble carbohydrates and proline in leaves took place simultaneously or preceded slightly the increases occurring in the flavedo, the leaves may be the direct source of soluble carbohydrates and proline in fruit during cold acclimation. Leaves have been shown to influence hardening of citrus stem tissue (12). Studies are needed to determine the influence of leaves on sugar and proline metabolism of the fruit during cold acclimation and the effect they have on development of resistance to chilling injury in fruit. In addition, the exchange of metabolites between the flavedo and other fruit parts, i.e. albedo tissue and juice vesicles, during cold acclimation should be studied.

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Growth and Respiration of Dormant Flower Buds of *Pyrus communis* and *Pyrus calleryana*[1]

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Additional index words. Prunus armeniaca, Prunus salicina, Prunus avium, Prunus domestica, Prunis serrulata, Malus hupehensis, Malus domestica

Abstract. **Respiration of flower-buds of *Pyrus communis* L., a late blooming species, and *P. calleryana*, an early blooming species, was investigated throughout the winter. Respiration of *P. calleryana* Decne at 5°C was twice as high as that of *P. communis*, whereas the respiration rates were similar at 25°. A large portion (60–70%) of the respiration at 5° was cyanide resistant in *P. calleryana* and much less in *P. communis*. The combination of inhibitors, cyanide (KCN) and salicylhydroxamic acid (SHAM), still only partially inhibited respiration. The residual respiration was much higher for *P. calleryana* than for *P. communis*. The nature of the residual respiration is not known.**

It appears that respiration of *P. calleryana* is less sensitive to cold than that of *P. communis*. *P. calleryana* also appears to complete the development of its buds during the winter and blooms early. In contrast, *P. communis*, the species which has a respiratory pathway less operational at relatively low temperatures, delays the completion of its buds until the spring when the temperature warms up, and consequently flowers later. This hypothesis was evaluated by measuring the mid-winter respiration of 5 early and 5 late blooming species of fruit trees, and the bloom date was evaluated relative to the type of respiration as described above.

Resting buds of fruit trees in general and pears in particular enter into dormancy with partially developed buds. The buds apparently complete their development by the time growth is resumed. The time of bloom varies from year to year and from species to species. There can be differences of up to one month for a species in a given location (2). The major factor regulating the time of bloom is temperature. A certain amount of cold is required to break the rest. After the cold requirement has been satisfied, a certain amount of heat is needed to enable the tree to bloom. It is conceivable that the time of bloom depends on the rate of development of buds during dormancy which in turn is influenced by temperature.

The time of bloom can be genetically transmitted (9) and intermediate bloom types created between early and late blooming pears. Generally the low chilling types (*P. calleryana*) also bloom early. The late blooming types (*P. communis*) bloom earlier when grafted on roots of early blooming types (*P. calleryana*) (24). Strausz (20) stated that this translocatable reduction of chilling requirement was due to a shift in growth promoters.

Pear buds are metabolically active during the winter. Thom (21) measured respiration of dormant buds of *P. communis* 'Hardy' and found that O_2 consumption increased six-fold when growth resumed in the spring. Zimmerman et al. (26) found that incorporation of uracil and valine into alcohol insoluble components of buds was high during the winter indicating that pear buds were not "resting" but metabolizing actively without visible in-

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