tion for a prediction of yield for a once-over harvested pickle crop. It is more important to have a supply of N available for the crop early in the season during initial plant development. Subsequent sidedressing of N will then only be beneficial in cases where maximum soil depletion of N occurs.

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Accumulation of Sugars and Plasmalemma Alterations: Factors Related to the Lack of Cold Acclimation in Young Roots[1]

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Abstract. **The possibility that low cellular concentration of sucrose was limiting the expression of hardiness in young roots of *Pyracantha coccinea* Roem. 'Lalandii' Dipp. was investigated. While the sucrose content of young roots increased four-fold following exposure to 4°C, the highest concentration was not higher than that found in non-acclimated mature roots. Attempts to increase hardiness by incubating young roots on sucrose solutions were unsuccessful. However, intracellular sucrose concentrations were not significantly increased by this treatment. Cytochrome oxidase incorporated into a membrane fraction containing plasmalemma vesicles isolated from *Pyracantha* young roots or from tissue capable of acclimation (*Hedera helix* L. 'Thorndale' callus cultures) was used as a probe for architectural alterations of this membrane following exposure to 4° and 5°. The apparent first order rate constant of the cytochrome oxidase reaction was used to indicate membrane fluidity. Above the Arrhenius discontinuity, membrane fluidity in both species was greatest when plants were grown at 4° or 5°. However, below the Arrhenius discontinuity fluidity remained greater in ivy callus grown at 5°, but not in *Pyracantha* young roots exposed to 4°. Altered properties of the membrane surface, inferred from the second order rate constant, were observed only in plasmalemma of young roots. Several possibilities to account for the lack of young root hardiness are presented.**

Studies of root hardiness of numerous woody plants have demonstrated that the root system is substantially less hardy than that of the shoot system (5, 20, 23, 38). The inability of young roots to survive extreme freezing temp is one of the prime factors limiting container production of woody ornamentals in northern regions. Previous work indicates that the intensity of the physical freezing stresses in both young and mature roots of *Pyracantha* declines when plants are grown at 4°C; however, the killing point of young roots remains unaffected while that of mature roots declines (38). Since the stress appears to be a near-equilibrium, low intensity type, attention is directed to the basic question of the acclimation process.

The primary site(s) of freezing injury is probably at cellular membranes (12, 15, 17, 29). Therefore cold acclimation must in some way prevent membrane injury. Heber (12) states that " . . . the ability of hardy cells to withstand freezing is not the result of a 'special' condition of their protoplasm but rather, the consequence of the presence of cryoprotectants." Steponkus (35) maintains that cold acclimation involves both the presence of cryoprotectants and a concomitant change in cellular membranes. Whether one or both of these factors are limiting root hardiness is unknown.

In work concerning the effect of freezing and cold acclimation on plant membranes, cellular organelles have been utilized rather than the plasmalemma itself. Since the exact mechanisms of freezing injury and cold acclimation are unknown, we elected to measure plasmalemma fluidity, a general parameter of plasmalemma architecture, to educe changes in the membrane following cold acclimation, rather than merely considering compositional changes in the membrane.

That membrane architecture can be manifested by functional properties of the membrane is currently a popular conception (10, 11, 26, 30, 33). However, functional properties of plant plasmalemma are not well defined at this time. To overcome this obstacle, exogenous proteins whose activity is dependent on association with lipids can be employed. The enzymatic activity of cytochrome oxidase, the terminal enzyme of the mitochondrial respiratory chain, is dependent upon its association with lipids (7, 13, 18). Sonication of cytochrome oxidase

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in the presence of phospholipids results in the formation of cytochrome oxidase vesicles (7, 13, 25). The activity of the lipid-depleted enzyme increases with added phospholipid until a phospholipid:oxidase ratio of about 50% w/w is attained (7). Essentially maximal fluidity, as indicated by spin-labelled probes, is apparent at this ratio (viz. Figs. 3 and 6, ref. 13). Although Jost et al. (13) present a different conclusion based on a single point of their activity measurements (viz. Fig. 9, ref. 13), other activity measurements under essentially the same conditions of phospholipid incorporation are more convincing (viz. Fig. 3, ref. 7) and the two sets of data combined (7, 13) support the contention that cytochrome oxidase activity is positively correlated with membrane fluidity.

Other indirect evidence to support this contention is as follows. Ainsworth et al. (1) demonstrated that cytochrome oxidase activity of *E. coli* grown in the presence of linoleic or oleic acid shows corresponding differences in the Arrhenius transition temp. That is, the transition temp is affected by the fatty acid environment of cytochrome oxidase. This Arrhenius transition is abolished by 0.05% sodium dodecyl sulfate or 0.1% triton X-100 (27). That this is not due to the dissolution of lipids tightly associated to cytochrome oxidase is suggested by even more severe extraction of mitochondria with 10% cholate, followed by 1 or 2 ammonium sulfate precipitations in 2% cholate, resulting in a lipid content of cytochrome oxidase of about 30% (40). The purported phospholipid binding sites of the oxidase are saturated at about 20% phospholipid (13). Green et al. (11) describe experiments wherein cytochrome oxidase particles were found to be arranged in regular arrays in the membrane when oxidized, and randomly scattered throughout the membrane matrix when reduced. Chance (4) has also observed that cytochrome *a* apparently requires lateral diffusion for activity. These data taken together strongly suggest, but no not prove, that cytochrome oxidase activity is dependent on membrane fluidity.

The purpose of this study was to determine if the inability of young roots of *Pyracantha* to acclimate was associated with either a limited accumulation of cryoprotectants or a lack of structural alterations in the plasmalemma. To this end, the carbohydrate status of *Pyracantha* tissues was examined following exposure to acclimating temp. In addition, the activity of cytochrome oxidase incorporated into plasmalemma vesicles of *Pyracantha* young roots was compared with that of the same enzyme incorporated into vesicles isolated from tissue which can undergo acclimation (*Hedera helix* callus).

Materials and Methods

Plant materials. Three-year-old *Pyracantha* plants were grown in 8.8 liter containers in a 1 soil:1 peat:1 perlite (by vol) mixture at 18°C in a greenhouse or in a 4° growth chamber as described elsewhere (38). Hardiness was determined by a modified ninhydrin test (39). "Stems" are defined as 1-year-old twigs, "mature roots" as woody primary roots 2 to 5 mm in diam, and "young roots" as fleshy white roots about 1 mm in diam and adjacent root tips. Callus cultures of ivy were grown on Murashige-Skoog medium (21). After subculturing, the cultures to be acclimated were grown at 27° for 10 days before being transferred to a growth chamber at 18° or 5°.

Attempts to artificially increase the sucrose content of young roots were made by shaking excised young roots at 100 rpm for 3 days at 4°C in water, 1.4 μM abscisic acid (ABA), 100 mM sucrose, or 1.4 μM ABA plus 100 mM sucrose. The roots were then washed twice in water (total washing time of about 1 hr) prior to the sugar analysis described below.

Accumulation of cryoprotectants. The carbohydrate status of *Pyracantha* tissues was examined by lyophylizing samples and grinding them to a 40 mesh powder in a Wiley mill. Samples were stored *in vacuo* at room temp until analysis. Samples (10 mg) were extracted with 4 ml 80% (v/v) ethanol for 10 hr at 65°C. The solution was decanted and the residue washed 3 times. Sample volumes were reduced *in vacuo* to 1 to 2 ml and brought to 5 ml with water. The samples were extracted 3 times with equal volumes of petroleum ether, and concentrated *in vacuo* to 1 to 2 ml before passing through columns of Dowex H^+ and Cl^-. The neutral effluent was brought to a final volume of 5 ml and an aliquot assayed for reducing sugars (22). Another aliquot was hydrolyzed in 0.08 N HCl at 100° for 1 hr, neutralized with 0.08 N NaOH and analyzed for reducing sugars (22). This procedure was found to completely hydrolyze sucrose and not degrade the fructosyl moiety. Approximately 60% of raffinose was hydrolyzed by this technique.

Qualitative identification of the sugar components was performed by multiple thin-layer chromatography. Plates were prepared from a slurry of 35% (w/w) silica gel G in 0.1 N boric acid and activated at 110°C for ½ hr immediately before running. Plates were run twice in butanol-acetone-water (4:5:1 by vol) and once in 2-butanone-acetic acid-methanol (3:1:1 by vol) with intermediate drying times of 1 hr. R_f values for raffinose, fructose, sucrose and glucose were typically 0.35, 0.50, 0.60, and 0.70, respectively. Spots were visualized with aniline-diphenylamine spray (32).

Plasmalemma alterations. Plasmalemma from *Pyracantha* young roots or *Hedera helix* 'Thorndale' tissues cultures were obtained essentially as described by Leonard et al. (14). Discontinuous sucrose gradients were centrifuged for 3 hr at an RCF_{max} of 83,000 × *g*. Membranes from the 34/38% (w/w) and 38/45% interfaces were combined and washed twice in 1 mM tris-MES, pH 7.2, 1 mM $MgSO_4$. Membranes were pelleted by centrifugation for 40 min at 83,000 × *g*. The pellet was finally resuspended in 15 mM potassium phosphate buffer, pH 7.0. This procedure yields ca. 45% pure plasmalemma from *H. helix* cultures, as determined by electron microscopy using a stain which is specific for plant plasmalemma (28). Protein was determined by the Folin-Ciocalteu procedure (2).

Cytochrome oxidase was obtained from mitochondrial preparations of bovine heart muscle (31) according to the procedure of Yonetani (41). The purified preparation was stored at −40°C until use. Activity was determined polarographically with a Clark-type O_2 electrode in a reaction medium of 50 mM potassium phosphate, pH 6.0, 1 mM EDTA, 25 mM sodium ascorbate and varying amounts of cytochrome oxidase and type VI cytochrome *c* (Sigma Chemical Co.) in final volumes of 3.011 to 3.055 ml. Temp was controlled with a circulating, refrigerated water bath. Steady state levels of reduced cytochrome *c* were spectrophotometrically determined at 550 nm and corrected for absorbance by oxidized cytochrome *c*.

Cytochrome oxidase kinetics essentially followed the equation described by Minnaert (19):

$$v = k_1 k_2 (c^{++}) (e) / k_1 (\text{total } c) + k_2$$

where v is the rate of oxygen uptake, (c^{++}) is the steady state concn of reduced cytochrome *c*, (total *c*) is the total concn of cytochrome *c*, and (e) is the oxidase concn. k_1 is analogous to V_{max}/K_m and k_2 is analogous to V_{max} in Michaelis-Menten terminology. The exact molecular mechanism of cytochrome *c* oxidation is, however, unknown.

Cytochrome oxidase-plasmalemma vesicles were prepared in a manner analogous to the cytochrome oxidase vesicles of Racker (25). The components were combined in a ratio of 1 mg plasmalemma protein to 40 nmoles oxidase. This mixture was then sonicated in a Bransonic 220 ultrasonic cleaner, 125 watts, for 5 min at room temp. Vesicles were incubated for 6 hr at 0°C before assaying.

Results and Discussion

Accumulation of cryoprotectants. Contrary to the results obtained by Preston and Phillips with 9 different species of forest trees (24), young and mature roots of *Pyracantha* increased in both reducing and non-reducing sugar content after exposure to 4°C for 5 weeks (Table 1). Thin-layer chromato-

Table 1. Effect of growth temp on sugar content of *Pyracantha* tissues.[z]

Tissue	Growth temp (°C)	Killing point (°C)	Reducing sugars (% dry wt)	Non-reducing sugars (% dry wt)
Young roots	18	−5	2.77b	0.42a
	4	−5	3.51c	1.74b
Mature roots	18	−8	2.74b	0.71a
	4	−17	3.94c	2.43b
Stems	18	−8	1.82a	0.58a
	4	−26	2.33ab	2.42b

[z]Values represent the mean of 3 replications. Mean separation within columns by Duncan's multiple range test (34), 5% level.

Table 2. Effect of incubation in 100 mM sucrose on sucrose accumulation by *Pyracantha* young roots.[z]

Growth temp (°C)	Treatment	Killing point (°C)	Sugar content (% dry wt)	
			Reducing sugars	Non-reducing sugars
18°	Water	−5	2.69a	0.68a
	100 mM sucrose	−7	3.20b	0.84ab[y]
4°	Water	−5	3.20b	1.35c
	100 mM sucrose	−5	3.73c	1.21bc[y]

[z]Values represent the mean of three replications. Mean separation within columns by Duncan's multiple range test (34), p = 5%.

[y]The non-reducing sugar content of roots incubated in sucrose was corrected for sucrose adsorption by subtraction of the non-reducing sugar content of dead roots incubated on sucrose. These values were 0.14 and 0.34% dry wt for roots at 18° and 4°, respectively.

graphy of the extracts revealed only glucose, fructose and sucrose in detectable quantities. Incubation of excised *Pyracantha* young roots on sucrose had no effect on the killing point and resulted only in the accumulation of reducing sugars (Table 2), again chromatographically identified as glucose and fructose. Chin and Weston (6) have observed an isozyme of invertase in tomato, inducible by sucrose, whose synthesis was inhibited by abscisic acid (ABA). Incorporation of ABA into the feeding solution, however, had no effect on either the killing point or accumulation of sugars by *Pyracantha* young roots (Table 3).

The question of whether the concn of sucrose (or some other cryoprotectant) is limiting the expression of potential hardiness of young roots is difficult to answer unequivocally. The 4-fold increase in non-reducing sugars following exposure of young roots to 4°C (Table 1) should have increased hardiness had sugars been limiting. However, if the concn is calculated on a molar basis (assuming 80% moisture) their concn increased from 3 mM to 13 mM. The concn in non-acclimated and acclimated mature roots was 14 and 47 mM, respectively. Steponkus (35) observed that incubating pre-starved *H. helix* cuttings on 50 mM sucrose increased hardiness whereas solutions of 1 to 25 mM had no significant effect on the killing point, although the amount of sucrose in the tissue was not determined. Unfortunately, such measurements reflect only the gross tissue content of sugars. Whether these values reflect the cytoplasmic content awaits development of methods for determining the intracellular localization of these sugars.

Table 3. Effect of ABA on sucrose accumulation by *Pyracantha* roots.[z]

Growth temp (°C)	Treatment	Killing point (°C)	Sugar content (% dry wt)	
			Reducing sugars	Non-reducing sugars
18°	ABA	−5	2.98a	0.78a
	ABA + sucrose	−6	3.53a	0.83a[y]
4°	ABA	−5	2.82a	1.36a
	ABA + sucrose	−4	3.15a	1.36a[y]

[z]Values represent the mean of three replications. Mean separation within columns by Duncan's multiple range test (34), p = 5%.

[y]The non-reducing sugar content of roots incubated in ABA + sucrose was corrected for sucrose adsorption by subtraction of the non-reducing sugar content of dead roots incubated on ABA + sucrose. The values were 0.40 and 0.75 % dry wt, respectively.

Alterations in ivy plasmalemma following cold acclimation. The ideal comparison to make would be between young roots and mature roots or stems of *Pyracantha,* that is, tissue which does not acclimate vs. tissue from the same plant which does (38). However, to our knowledge plasmalemma have never been isolated from woody tissues such as mature roots and stems. Therefore, plasmalemma alterations following acclimation were investigated with ivy callus cultures. When grown at 18°C and frozen to −6°, the increase in fresh wt after 4 additional weeks at 18° was 50% that of the unfrozen control. However, when calluses were grown at 5° for 4 weeks before freezing to −15°, further regrowth at 18° was 100% of the control, demonstrating that acclimation had occurred.

Cytochrome oxidase-plasmalemma vesicles were prepared from acclimated and non-acclimated callus cultures of *Hedera helix* 'Thorndale'. Maximal molecular activity (k_2) was consistently higher in vesicles prepared from acclimated calli than in those prepared from non-acclimated calli (Fig. 1). Breaks in the Arrhenius plots occurred at about 22°C ($10^3/T$ = 3.39° K^{-1}) for vesicles prepared from acclimated tissue vs. about 19° ($10^3/T$ = 3.42° K^{-1}) for vesicles prepared from non-acclimated tissue. Note that the increased fluidity in vesicles from acclimated tissue appears to be maintained both above and below the break in the Arrhenius plot. However, analysis of variance indicates that points at $10^3/T$ = 3.47° K^{-1} and 3.50°

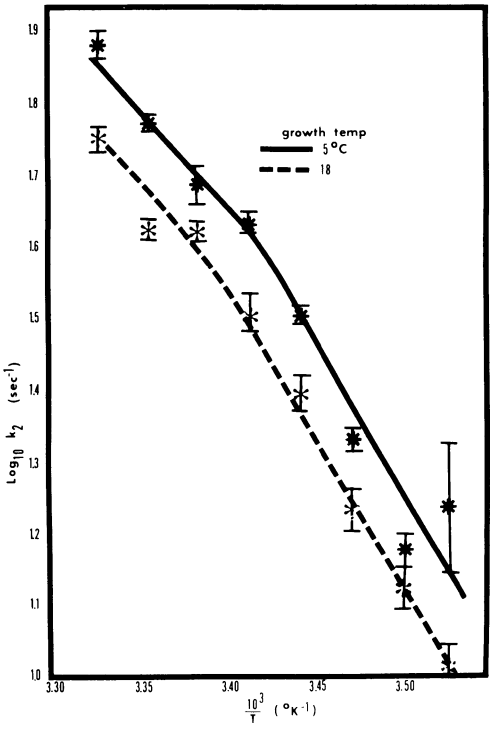

Fig. 1. Arrhenius plots of first order rate constants (k_2) obtained from oxidase-plasmalemma vesicles prepared from ivy callus grown at 18° or 5°C for 10 weeks. k_2 values were calculated by least squares regression analysis of oxygen uptake rates obtained from 5 different oxidase concn at 5 different concn of cytochrome *c*. Each line comprises data of a single plasmalemma isolation. Bars represent standard errors.

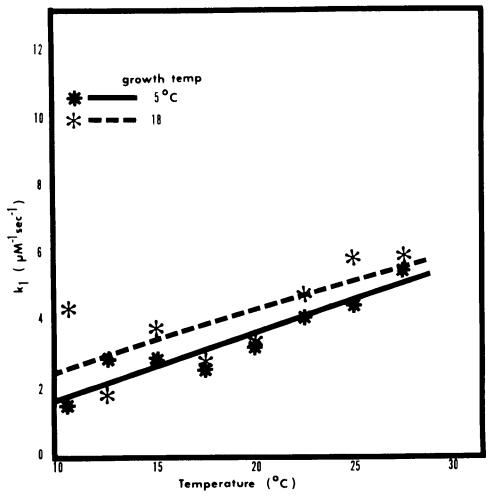

Fig. 2. Temp dependence of the second order rate constant (k_1) obtained from oxidase-plasmalemma vesicles prepared from ivy callus grown at 18° or 5°C for 10 weeks. k_1 values were calculated by least squares regression analysis of oxygen uptake rates obtained from 5 different concn of cytochrome *c* at an oxidase concn of 66.7 nM. Each line comprises data of a single plasmalemma isolation. Correlation coefficients of both lines are significant at the 5% levels. Analysis of variance indicates no significant difference between the regression lines either in the slope or in the height at the 5% level.

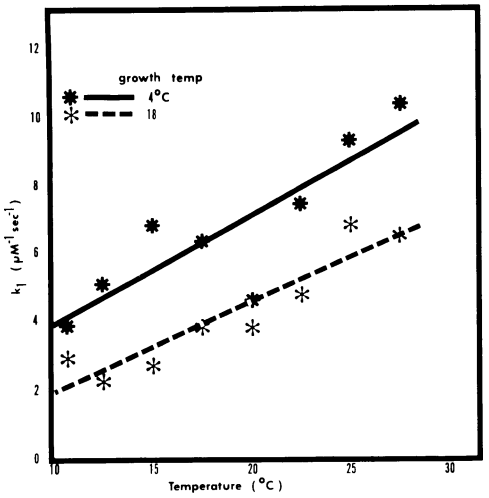

Fig. 4. Temp dependence of k_1 values obtained from oxidase-plasmalemma vesicles prepared from *Pyracantha* young roots grown at 18° or 4°C for 10 weeks. k_1 values were calculated as described in Fig. 2. Each line comprises data of a single plasmalemma isolation. Correlation coefficients of both lines are significant at the 5% level. Analysis of variance at the 1% level indicates that k_1 values are significantly larger in vesicles prepared from tissue grown at 4° than in those grown at 18°. The slope of the regression lines are not significantly different at the 5% level.

K^{-1} are not significantly different at p = 5%. All other values for acclimated ivy tissue are significantly higher than those for non-acclimated tissue (p = 1%). The temp of breaks in Arrhenius plots has been correlated with changes in the saturation of fatty acids (1) and may represent phase transitions (26), although such possibilities as conformational changes in the protein cannot be excluded. Membrane fluidity can also be altered by changes in the interior or exterior portions of the membrane since fatty acids (16), sterols (37), and proteins (3) influence fluidity. Gitler (10) discusses several ways by which bound external molecules can affect head groups of polar lipids, altering membrane fluidity.

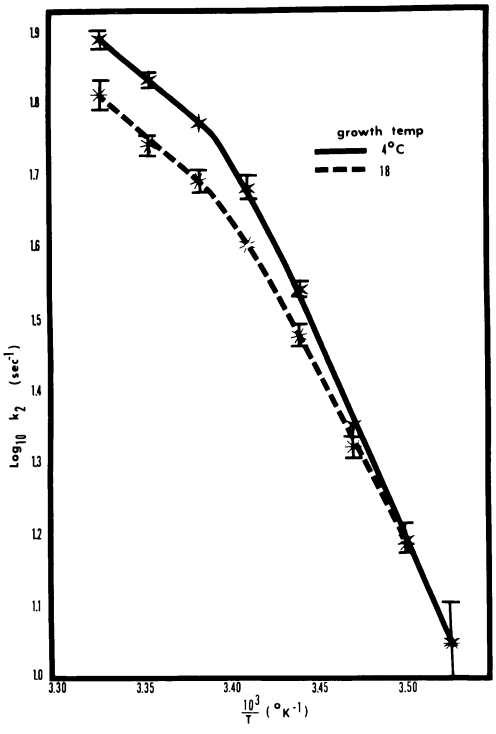

Fig. 3. Arrhenius plots of k_2 values obtained from oxidase-plasmalemma vesicles prepared from *Pyracantha* young roots grown at 18° or 4°C for 10 weeks. k_2 values were calculated as described in Fig. 1. Each line comprises data of a single plasmalemma isolation. Bars represent standard errors and have been omitted when the standard error is smaller than the symbol.

It can be postulated that, while k_2 values reflect kinetic factors of the membrane such as fluidity, the interaction of cytochrome oxidase with cytochrome *c* will be influenced by electrical properties (8) and the three-dimensional topography of the membrane surface. The interaction of cytochrome *c* with cytochrome oxidase can be inferred from values of the second order rate constant, k_1. No differences in k_1 were evident between acclimated and non-acclimated tissue (Fig. 2).

Specific interpretations of these results are highly speculative, but the plasmalemma vesicles obtained from *Hedera helix* callus are obviously altered following cold acclimation. It was therefore desirable to determine whether similar alterations occurred in young roots of *Pyracantha.*

Alterations in Pyracantha root plasmalemma following exposure to 4°C. Unlike the results obtained with plasmalemma of ivy callus, the temp at which the break in the Arrhenius plot occurs does not vary with growth temp of *Pyracantha* young roots (Fig. 3). Regardless of the growth temp the discontinuity occurred at about 22° ($10^3/T = 3.39°\ K^{-1}$), the same temp as for non-acclimated *Hedera helix* tissue. Oxidase activity (k_2) is significantly higher (p = 1%) in vesicles prepared from plants grown at 4° above the transition temp but the difference decreases below this temp. Points at $10^3/T = 3.44°\ K^{-1}$ and greater (i.e., lower temp) are not significantly different at p = 1%. Thus response of *Pyracantha* root tissues parallels that of ivy callus above the transition temp, but below the transition temp, increased fluidity was not evident as it was in ivy tissue.

The second order rate constant (k_1) for plasmalemma of roots grown at 18°C was similar to that observed for plasmalemma of ivy callus, but was significantly higher (p = 1%) in vesicles prepared from roots grown at 4° (Fig. 4). Phospholipids are believed to facilitate the interaction of cytochrome *c* and the oxidase (7, 8, 13, 36). Alternatively, the higher rate constant could result from a more favorable membrane topography due to either a more highly charged environment around cytochrome *a* or fewer neighboring extrinsic molecules to sterically hinder the approach of a cytochrome *c* molecule.

As it is generally accepted that freezing injury is the result of membrane disruption, it follows that cold acclimation results in a mitigation of membrane disruption either by alterations in the membrane environment, by alterations in the membrane itself, or a combination of both. Heber (12) considers an increase in cryoprotective compounds to solely account for acclimation, Levitt and Dear (15) have speculated that there is an altered membrane protein composition, while Sakai and Yoshida (29) and Steponkus (35) consider both changes in the cellular environment and membrane alterations to be required. This latter view is supported by recent electron microscopy and biochemical data (9).

The results presented herein demonstrate that both reducing and non-reducing sugars in *Pyracantha* young roots increase after exposure to 4°C. The plasmalemma-rich membrane fraction of *Pyracantha* young roots is also altered following exposure to 4°, but in a manner different from plasmalemma alterations observed in ivy tissue cultures following acclimation.

It is presently unclear which of several possibilities account for the lack of young root hardiness. The observed membrane alterations may be causally related to the acclimation process but do not elicit a change in the killing point because the sucrose concn is not above some critical level necessary for the manifestation of hardiness. Alternatively, the alterations in the plasmalemma of young roots may not suffice for efficient interaction with sucrose. A third possibility, obtained from the evidence that the interaction between cytochrome *c* and the oxidase is altered in vesicles prepared from young roots grown at 4°C, is that the surface of the plasmalemma of young roots is altered upon exposure to 4°, and this alteration prevents the expression of membrane properties required in acclimated tissues.

However, interpretation of these results is not unambiguous. For instance, quantitation of tissue sugar content does not exclude possible differences in its subcellular localization. Similarly, cytochrome oxidase activity may be affected by impurities in the plasmalemma preparations. In addition, the *in vitro* state of the membranes may not resemble their *in vivo* state. While these problems preclude the precise identification of membrane alterations related to cold acclimation, they nevertheless document a "change" in the plasmalemma following acclimation.

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