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Physiological Aspects of the Cold Hardiness

of <u>Ledum groenlandicum</u>

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Physiological Aspects of the Cold Hardiness of Ledum groenlandicum", submitted by Amanda June Wilkinson in partial fulfilment of the requirements for the degree of Master of Science in Botany.

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# ABSTRACT

The survival temperature of <u>Ledum groenlandicum</u> growing at the University of Alberta Devonian Botanic Gardens 25 km SW of Edmonton, Alberta varied from -9 C in the summer of 1976 to <-40 C in the winter of 1976-77. Controlled environment studies suggested that <u>Ledum groenlandicum</u> requires two stages of cold acclimation. The first stage of acclimation requires a short day (8 h) photoperiod and above freezing temperatures. The second stage of acclimation is triggered by night frosts (eg. 2 h at -2 C).

Throughout the summer and early fall of 1976, <u>Ledum groenlandicum</u> leaf water potential ($\Psi$) was above -25 bars and the combined osmotic and matric potential ($\Psi_{\pi+\tau}$) was above -30 bars. Turgor pressure ($\Psi_p$) was positive. Examples of summer and early fall hydration levels are as follows: on July 21, 1976 $\Psi$ was -11.7 bars, $\Psi_{\pi+\tau}$ was -16.2 bars, and $\Psi_p$ was 4.5 bars; on September 25, 1976 $\Psi$ was -10.8 bars, $\Psi_{\pi+\tau}$ was -14.0 bars, and $\Psi_p$ was 3.2 bars. Water potential values decreased with the occurrence of October frosts. Winter water potential was regularly below -67.5 bars and turgor pressure was negative. In controlled environment studies, a short day photoperiod during cold acclimation caused a decline in tissue hydration whereas a long day photoperiod had no effect.

The xylem sap of <u>Ledum groenlandicum</u> was found to

cavitate following artificial freezing. No cavitation following freezing was found in the xylem sap of _Pinus banksiana_. The solute and colloidal concentration was found to increase with hardening in _Ledum groenlandicum_. Analysis of Höfler diagrams suggested that cell wall elasticity decreases and the proportion of apoplastic water increases in hardened _Ledum groenlandicum_. Hardened _Ledum groenlandicum_ had a coefficient of enlargement of 65.2 bars compared to 36.6 bars for unhardened tissue.

The transpiration rate of hardened _Ledum groenlandicum_ was $139 \pm 20$ mg $g^{-1}$ dry wt $h^{-1}$ in the light compared to a rate of $242 \pm 26$ mg $g^{-1}$ dry wt $h^{-1}$ for unhardened shoots. In the dark, the transpiration rate of hardened _Ledum groenlandicum_ was $103 \pm 16$ mg $g^{-1}$ dry wt $h^{-1}$, about 3 times higher than the rate of unhardened shoots.

The maximum net photosynthetic rate of unhardened _Ledum groenlandicum_ under optimal conditions was $4.06 \pm .20$ mg $CO_2$ $dm^{-2}$ $h^{-1}$. Hardened plants had a reduction in their photosynthetic capacity. The maximum recorded net photosynthetic rate was $1.77 \pm .50$ mg $CO_2$ $dm^{-2}$ $h^{-1}$. _Ledum groenlandicum_ exhibited thermal acclimation to the daytime growing temperatures. There was an increase in the respiratory capacity of hardened _Ledum groenlandicum_. Interpretations of an Arrhenius plot revealed that neither unhardened nor hardened plants were chilling sensitive.

## ACKNOWLEDGEMENTS

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## INTRODUCTION AND LITERATURE REVIEW

Drought and cold are generally considered to be the two most important climatic factors affecting plant distribution. For a plant species to survive, it must be capable of developing a resistance to a temperature that is below the minimum temperature of its ecological range. Plants are poikilotherms, therefore, they must tolerate, rather than avoid, subfreezing temperatures. Northern deciduous plant species partially avoid subfreezing temperatures by seasonally shedding their foliage. The foliage of northern conifers and broadleaf evergreens is exposed to the prolonged subfreezing winter temperatures. However, the evergreen habit provides a readily available energy source in the spring, and thus, is advantageous in the relatively short growing season of the northern clime (Scholander et al. 1953).

Ledum groenlandicum is an evergreen, ericaceous shrub ranging from the NW coast of Oregon, northward into the Olympic Peninsula, British Columbia, and possibly northern Idaho, extending north to Alaska, and eastward into Greenland and N W England (Hitchcock et al. 1977). Throughout its range, Ledum groenlandicum inhabits acid, rather than moist, soils (Hitchcock et al. 1977) and is commonly found in muskegs and moist coniferous woods (Moss

1967).

Ledum groenlandicum is an erect shrub from 3 to 8 dm high. It exhibits a growth form that is typical of most members of the Ericaceae. Its leaves have a thick-walled, single-layered epidermis that is covered with a thick cuticle. The lower surfaces of the leaves and twigs are rusty-tomentose. Stomata are confined to the lower leaf surface. The leaf anatomy of Ledum groenlandicum is peculiar: it has xeromorphic adaptations, that include a thick cuticle and a closely packed palisade layer; yet it also has hydrophilic adaptations, such as large airspaces that occupy a considerable portion of the mesophyll (Metcalfe and Chalk 1957).

It has been suggested that the xeromorphic adaptations of the Ericaceae are a result of a 'physiological drought' imposed upon the plant even in times of high water availability. Gates (1914) and Small (1972) suggested that the xeromorphic adaptations, including sclerophylly, small cell size, a well developed cuticle, revolute leaf margins, coverings of hairs, sunken stomata, and a waxy bloom, all contribute to the plant's resistance to winter drought. Observations of the growth habit of overwintering members of the Ericaceae support the idea that a plant experiences dehydration during periods of subfreezing temperatures. The leaves of Rhododendron catawbiense were found to droop and curl from January to May (Havis 1966). Gates (1914) and Bannister (1964) observed an increase in the dark red

pigmentation (presumably anthocyanins) in exposed ericaceous foliage during the winter. An increase in anthocyanins during cold acclimation was also observed in _Hedra helix_ (Parker 1962), however, no causal relationship between the anthocyanin level and an increased cold hardiness was found (Steponkus and Lanphear 1969).

In the majority of plants exposed to subfreezing temperatures, the cell water has been found to freeze and the freezing resistance of plants is usually considered a tolerance mechanism to the ice formation. However, supercooling, i.e. the avoidance of ice formation in cooled tissue, has occasionally been observed. Pellet and White (1969) suggested that water binding substances in the roots of _Juniperus_ _chinensis_ may have caused significant supercooling. Lumis, Mecklenburg, and Sink (1972), Milon, Burke, and Weiser (1974), Graham and Mullin (1976a), and George and Burke (1977a) found no sign of ice formation in the floral primordia of azalea. In azalea, ice existed in the stem axis and bud scales at -10 C, therefore, George and Burke (1977a) suggested that a physical barrier, or thermodynamic equilibrium, must have existed between the tissues to prevent the growth of ice into the supercooled floral primordia. Ice was found in the extremely hardy stem tissue of _Cornus_ _stolonifera_ (Weiser 1970), however, none was found in the living bark of shagbark hickory (George and Burke 1977b) or in several other hardwood species native to the eastern deciduous forest (Burke, George, and Bryant

1975). The supercooling of water in the living xylem depended upon the isolation of the rays preventing the propagation of the ice or vapour cavitation from other tissues.

The freezing of a solution can be delayed if its freezing point is lowered. An increased sugar and salt concentration in hardened tissue, causing a depression in the cell sap freezing point, has been suggested as a freezing avoidance mechanism (eg. Havis 1973). However, an osmotic potential of -50 bars would lower the freezing point of the cell sap solution to only -4 C. Freezing point depression, therefore, cannot solely account for the freezing resistance of cold hardened tissue.

Because freezing avoidance mechanisms cannot explain the extreme freezing resistance found in woody plants, a freezing tolerance mechanism has been sought. Intracellular freezing causes the formation of large ice masses within the protoplast, and thus, results in a lethal mechanical injury to the frozen plant cell (eg. Levitt 1972). Intracellular freezing has been found to occur in rapidly frozen cold hardened tissue. Hardened Rhododendron catawbiense leaves were killed when they were frozen rapidly to -18 C (Havis 1964). The branches of Nothofagus procera exposed to a wide range in temperatures suffered more damage than those exposed to equally low minima but cooler day temperatures (Nimmo 1965). When branches of Thuja occidentalis were cooled at 17 C min⁻¹, intracellular freezing occurred. It

was found that the predominance of injury occurred on branches on the SW side of the plant exposed to the rapid temperature fluctuations at sunset (White and Weiser 1964).

Because of freeze-desiccation, freezing can injure hardened woody plants even if intracellular freezing does not occur. Freeze-desiccation is caused by the loss of water from thawed leaves at a time when the translocation of water throughout the plant is impeded (eg. Levitt 1972). The impedance of translocation could be due to a resistance to water uptake in frozen soils or a resistance to water transport through cavitated xylem. Wilner (1952) found that freeze-desiccation occurred in the twigs of Caragana aborescens, Fraxinus pennsylvanica, Ulmus americana, Acer negundo, and Populus deltoides during mild spells in the winter. The N or NE branches of Cupressus macrocarpa, Chamaecyparis lawsonia, and Thuja plicata suffered injury from desiccating cold winds (Phillips 1965). White and Weiser (1964) observed 'winter burn' in the exposed branches of Thuja occidentalis under dry atmospheric and high radiant energy conditions accompanied by long periods of frozen soil. Desiccation of Rhododendron catawbiense leaves was observed late in winter even though the roots were in moist, but cold soil (Havis 1960). Sakai (1970) observed desiccation injury in several species of conifers overwintering in frozen soil areas in Japan. He also attributed the 'brown belt' phenomenon found in Chinook areas of North America to freeze-desiccation.

The extracellular freezing process that occurs in plant cells under the normal winter freezing rates of 3 to 5 C per hour (eg. Havis 1973), does not result in injury to hardy woody cells (Weiser 1970). The events of extracellular freezing are described both by Mazur (1969) and Levitt (1972) as follows:

1) both the cells and external medium initially supercool,

2) with a decrease in temperature, ice nucleation sites form in the large diameter vessels (having dilute sap) and spread through the intercellular spaces,

3) the cell membrane bars the seeding of ice crystals in the cell interior, so the cell water remains unfrozen and supercooled,

4) the aqueous vapour pressure of the supercooled cell is greater than the extracellular water, and water diffuses to the intercellular ice mass,

5) the resulting dehydration causes the cell sap concentration to rise, and lowers its aqueous vapour pressure,

6) if the membrane is sufficiently permeable to water and the tissue is cooled slowly, the cell will dehydrate until an equilibrium between the intracellular and extracellular vapour pressures exists,

7) if the tissue is cooled rapidly, or if the cell membrane is of low permeability, an equilibrium

between the extracellular and intracellular vapour pressures cannot be maintained and intracellular freezing will occur.

The freezing tolerance mechanism must involve an avoidance of intracellular ice formation and a tolerance of extracellular freezing and the accompanying secondary freeze dehydration. Hardy-plant tissue has been found to withstand a greater degree of freeze-dehydration than tender tissue. Gusta, Burke, and Kapoor (1975) found that tender winter wheat was killed when 18% of the tissue water had been removed from the protoplast. Hardened winter wheat was able to withstand the removal of 85% of the protoplastic water.

An interesting phenomenon was found to accompany the extracellular freezing process in Buxus leaves. 'Frostblase' (or large ice masses) were formed in the mesophyll intercellular spaces. The 'Frostblase' were typically around the layer of veins beneath the palisade layer and caused the separation of the spongy mesophyll from the veins (Hatakeyama and Kato 1965). The anatomical description of the spongy mesophyll layer of the Buxus leaf was similar to that given for Ledum groenlandicum by Metcalfe and Chalk (1957).

The freezing resistance (or cold hardiness) of several plant species has been determined with a variety of techniq Among the reported viability tests for cold stre e are; the quantitative determination of amino acids a ninhydri reacting substances liberated

after thawing (Siminovitch et al. 1964), neutral red uptake (Siminovitch et al. 1964), the identification of exotherms from cooling curves (McLeester, Weiser, and Hall 1968, Graham and Mullin 1976b), the reduction of triphenyl tetrazolium chloride (Steponkus and Lanphear 1967a, Yelenosky 1975a), and the xylem tension and chlorophyll fluorescence of frozen seedlings (Brown et al. 1977). An evaluation of several viability tests was given by Stergios and Howell (1973).

Plants growing in frost-prone areas develop a cold tolerance when they are subjected to freezing temperatures. The cold hardiness of the bark tissue of several _Citrus_ species, that were native to areas having sporadic frosts, was -6.7 C after the plants were grown under a 0 C thermal regime (Yelenosky and Guy 1977). As the range of a species extends into colder areas, its ability to cold harden increases. _Buxus microphylla_ leaves hardened to -35 C (Gusta and Weiser 1972) under a low temperature regime, _Rhododendron catawbiense_ and _Kalmia latifolia_ leaves naturally hardened to -30 C and -60 C respectively by February (Parker 1963a), and _Cassiope lycopodoides_, _Rhododendron aureum_, and _Ledum palustre_ leaves hardened to -40 C, -50 C, and -70 C respectively during the winter (Sakai and Otsuka 1970). Fully hardened stem cortex tissue of _Cornus stolonifera_ (Weiser 1970), living bark tissue of _Robinia pseudoacacia_ (Siminovitch et al. 1968), and black currant (Tumanov, Kuzina, and Karnikova 1965) survived

immersion in liquid $N_2$ (-196 C).

Plants are known to undergo seasonal variation in cold hardiness. In the studies by Sakai and Otsuka (1970) and Parker (1963a) listed above, the summer cold hardiness of the species was found to be between -5 C and -7 C. Unhardened Cornus stolonifera and black currant had a freezing resistance of -3 C (Chen, Li, and Burke 1977) and -5 C (Tumanov et al. 1965) respectively.

The characteristic seasonal changes in the cold hardiness of hardy woody species has been described as a two stage process of acclimation by Weiser (1970). Table 1 outlines Weiser's (1970) hypothesis for cold acclimation in hardy woody plants. Included in Table 1 is a third stage of acclimation. Weiser (1970) hypothesized that a third stage of acclimation is induced by prolonged low temperatures (-30 to -50 C). He stated that this stage is not commonly attained in nature and can be lost quickly with slight increases in temperature. Because of the transitory nature and the difficulty in detecting the third stage of acclimation, the majority of studies, including the present study, have been concerned only with the first two stages of acclimation.

Plants must stop growing before they undergo cold acclimation (eg. Tumanov et al. 1965, Weiser 1970). McKenzie, Weiser, and Burke (1974a) found evidence that natural endogenous rhythms influenced the ability of plants to effectively harden under controlled environment

Table 1. A hypothesis for the time and the events of cold acclimation in hardy woody plants (from Weiser 1970).

| Spring | Summer | Early Autumn | Late Autumn | Winter |
|---|---|---|---|---|
| tender | tender | first stage of acclimation | second stage of acclimation | third stage of acclimation |
| rapid growth | slow growth | growth stops | dormant | dormant |
| long days increasing temperature | long days warm temperature | short days warm days/ cool nights | short days frost | prolonged subfreezing temperatures |
| hardiness inhibitor produced | hardiness inhibitor produced | hardiness promoter synthesized | | |
| | | active metabolic changes | | |
| | | augmentation of protoplasm | augmentation of protoplasm | |
| | | reduced hydration | cellular components assume stable configuration | |
| | | increased membrane permeability | protoplasm elastic and resistance to dehydration | cellular water highly ordered and bound |

conditions. Plants that were taken indoors in winter, and thus, were in a state of physiological rest, were not responsive to controlled environment photoperiod regimes. The role of dormancy in the cold hardening process is not clear. A dormant condition in _Acer negundo_ and _Viburnum_ helped maintain the plants' cold hardy condition when they were exposed to dehardening temperatures of 21 C. When dormancy was broken by a sufficiently low temperature treatment, but not when broken by gibberellins, dehardening readily occurred. The dormant condition did not control the intial hardening or rehardening (Irving and Lanphear 1967).

The first stage of acclimation (Weiser 1970) is induced by a short photoperiod and above freezing temperatures. The short day requirement for the induction of the first stage of acclimation has been well documented for _Cornus stolonifera_ (Hurst, Hall, and Weiser 1967, van Huystee, Weiser, and Li 1967, Fuchigama, Evert, and Weiser 1971, McKenzie et al. 1974a, Chen et al. 1977), _Acer negundo_ (Irving and Lanphear 1967, Irving and Lanphear 1968, Irving 1969) and many other cold hardy species. However, Gusta and Weiser (1972) found that the leaves of Korean boxwood hardened to -35 C, regardless of the photoperiod, and low temperatures appeared to be the principal hardiness promoting factor. It has been implicated that the short day photoperiod initiates a phytochrome response, in which the production or activation of a translocatable hardiness promoter is stimulated.

The role of hormones in the first stage of acclimation
has not been verified, but it is accepted that the
translocatable hardiness promoter may be abscisic acid
(ABA). Many of the physiological changes that occur with
cold hardening have been found to be regulated by ABA.
Abscisic acid levels were found to increase with the onset
of dormancy in plum seeds (Lin and Boe 1972), birch
(Harrison and Saunders 1975), and several woody deciduous
species (eg. Wareing and Saunders 1971). Abscisic acid was
found to increase the membrane permeability (important in
the avoidance of intracellular freezing) in carrot tissue
(Glinka and Reinhold 1972), tomato roots (Glinka 1973), and
maize roots (Collins and Kerrigan 1974). The dehydration
experienced by overwintering plants may cause an increase in
ABA. Several plants have shown a rapid rise in ABA levels
after a water stress (Zeevart 1971, Hemphill and Tukey 1975,
Boubissa and Richmond 1976, Wright 1977). Alvim, Hewett, and
Saunders (1976) found an increase in the concentration of
ABA in the xylem sap of _Salix viminalis_ with the onset of
dormancy and cold hardiness. Exogenous applications of ABA
increased the cold hardiness of _Acer negundo_ (Irving 1969),
apple seedlings (Pieniazek and Holubowicz 1973), _Medicago
sativa_ (Waldman et al. 1975), and the chill resistance of
_Cucumis sativa_ (Rikin and Richmond 1976). Abscisic acid was
a common factor in causing increases in the drought and cold
tolerance of barley and tobacco leaves (Rikin and Richmond
1975). However, Young (1971) found that exogenous ABA had no

effect on the cold hardiness of <u>Citrus</u>. Steponkus and Lanphear (1967b) proposed that sucrose was the translocatable hardiness promoter in <u>Hedra helix</u>.

According to Weiser (1970) plants must remain photosynthetically active throughout the first stage of acclimation. Yelenosky and Guy (1977) found that <u>Citrus</u> did not harden in the dark or when the stomata were closed. When <u>Hedra helix</u> was grown in total darkness it did not increase in cold hardine (Steponkus and Lanphear 1967b). Yelenosky (1975b) found that a severe water deficit (-28 to -34 bars) and the application of an antitranspirant to the leaves of <u>Citrus</u> during the first stage of cold acclimation reduced the final cold hardiness. These conditions would have inhibited the photosynthetic production of sugars that accompanied cold hardening.

Freezing temperatures also inhibit the short day induced stage of cold acclimation (Weiser 1970). The effect of freezing temperatures in the first stage of acclimation would be to inhibit photosynthesis. Pharis, Hellmers, and Schuurmans (1970) found that after <u>Pinus ponderosa</u> and <u>Pseudotsuga menziesii</u> were subjected to a single night of subfreezing temperatures ranging from -4 C to -15 C, the recovery to the initial photosynthetic rates at 3 C took from 6 to 60 days. Photosynthetic rates were reduced to 20% of the pre-freeze rates when hardening <u>Abies alba</u> was exposed to -6 C (Bauer, Larcher, and Walker 1974). By December, when <u>Abies alba</u> was fully hardened, a temperature

of -12 C was required to depress the photosynthetic rate to
20% of the intial rate. Fully hardened Douglas fir seedlings
could also photosynthesize following subfreezing night
temperatures (Kreuger 1967). Table 2 gives the summer and
winter minimum temperatures for net photosynthesis for a
number of cold hardy plant species.

Subfreezing temperatures trigger the second stage of
acclimation, and it is during this stage that the rapid
increase in hardiness occurs (Weiser 1970). Temperatures
from -3 to -5 C were found to be the most effective
hardening regimes for Citrus (Yelenosky and Guy 1977). A few
days after the first frosts, Cornus stolonifera hardened
dramatically from -18 to -50 C (van Huystee et al. 1967).

Weiser (1970) suggested that the first stage of
acclimation initiates a series of metabolic changes within
the plant that ready it for the subfreezing temperatures
during the second stage of acclimation. Morphological and
anatomical changes resulting from the first stage of
acclimation have been related to the development of cold
hardiness. Roberts (1967) found that cold hardened winter
wheat varieties were stunted because the production of
smaller cells led to a reduction in organ size.

The majority of studies that have investigated the
metabolic changes during the first stage of acclimation have
dealt with physiological factors. An increase in the cell
sap concentration has been found to accompany cold hardening
in many species (eg. Levitt 1972, Havis 1973). The increased

Table 2. The minimum temperature for postive net
photosynthesis in some cold hardy plants (from Bauer,
Larcher, and Walker 1974).

| Plant Species | Minimum Temperature C | |
|---|---|---|
| | Summer | Winter |
| Coniferous Trees | | |
| Pinus sylvestris | -3.5 | -7.0 |
| Abies alba | -3.5 | -7.0 |
| Picea abies | -4.1 | -6.5 |
| Taxus baccata | -4.9 | -8.0 |
| Evergreen Trees | | |
| Laurocerasus officinalis | -4.0 | -6.0 |
| Pittosporum tobira | -4.0 | -7.5 |
| Citrus limon | -1.3 | -6.0 |
| Shrubs | | |
| Viscum album | -3.0 | -7.0 |
| Hedra helix | -3.0 | -7.1 |

solute concentration has been attributed to an increase in the soluble sugars of the cell sap. An increase in soluble sugar concentration with hardening was found in _Hedra helix_ leaves (Parker 1962), _Rhododendron catawbiense_ leaves, _Kalmia latifolia_ leaves, _Pinus nigra_ needles (Parker 1963a), _Juniperus chinensis_ shoots (Pellet and White 1969), _Robinia pseudoacacia_ bark (Siminovitch et al. 1968), _Triticum aestivum_ crowns (Green and Ratzlaff 1975), and _Citrus_ bark (Yelenosky and Guy 1977). Although the soluble sugar concentration has been found to increase with tissue hardiness in the autumn, the relationship of the two events is still not clear. Pellet and White (1969) fou that the sugar concentration in _Juniperus chinensis_ tissue continued to increase during the winter, whereas the tissue hardiness did not. Levitt (1972) stated that hardy plants can survive much lower temperatures than an increased sugar content can explain by means of a freezing point depression.

The protective role of sugars, or other substances acting as water binding agents, in the avoidance of freezing injury has been proposed. Water binding agents would inhibit the dehydration of the protoplasm during the movement of cellular water to extracellular ice formation sites (Pellet and White 1969). A glycoprotein that was ated from _Cornus florida_ accounted for 5 % to 7% of the cell sap and rendered 35% of the cell water osmotically nactive (Williams 1972). However, the isolation and the role of water binding agents in most cold hardy species has not been

established and a tolerance mechanism to the secondary freeze-dehydration seems more probable. Hardy winter wheat was found to withstand a greater percentage of its water being frozen than tender winter wheat, therefore, had a greater tolerance to the secondary freeze-dehydration (Gusta et al. 1975).

An increase in the membrane permeability of hardened tissue is considered an integral factor in the avoidance of intracellular freezing (eg. Mazur 1969). Stout and Steponkus (1973) found no increase in the membrane permeability of cold hardened Hedra helix, however, this may be an exceptional case. McKenzie et al. (1974c) found that the membrane permeability of cold hardened Cornus stolonifera increased. Changes within the protein and lipid fractions of hardening tissues, therefore, have received investigation in many species.

Changes in both the membrane-bound and the soluble proteins of cold acclimating tissue have been monitored. Increases in the concentration of soluble proteins with hardening have been found in Hedra helix leaves (Parker 1962), Robinia pseudoacacia bark (Siminovitch et al. 1968, Brown and Bixby 1975), Juniperus chinensis shoots (Pellet and White 1969), and Cornus stolonifera bark (Chen and Li 1977). However, an increase in the soluble proteins of apple seedlings was not accompanied by an increase in cold hardiness (Pieniazek and Holubowicz 1973). Qualitative changes in the the soluble proteins have also been found in

hardening tissues. Several new proteins were separated by electrophoresis in cold hardening alfalfa (Faw and Jung 1972). Seventeen new proteins appeared in the bark of cold hardening black locust seedlings (Bixby and Brown 1975). A new protein that appeared in cold acclimating Robinia pseudoacacia bark was identified as a glycoprotein (Brown and Bixby 1975), and thus, may have acted as a water binding agent. Roberts (1967) found a change in the temperature coefficient of invertase in cold hardened winter wheat leaves. He concluded that the hardening growing temperatures had altered the proportions of the invertase isozymes; the isozyme that was predominant in cold hardened tissue had a lower activation energy.

An increase in the insoluble (or membrane-bound) proteins of hardened tissue was found in hardening Robinia pseudoacacia bark (Siminovitch et al. 1968). There was a slight increase in the protein nitrogen fraction of the mitochondria, microsomes, and microsomal membranes. However, Brown and Bixby (1975) found no quantitative change in the insoluble protein fraction of hardening Robinia pseudoacacia bark.

Lyons (1972) hypothesized that the membranes of chill sensitive plants undergo a phase transition at 10 to 12 C, where the liquid crystalline lipid portion of the subcellular (particularly mitochondria) membranes becomes a solid gel. The lipid phase change supposedly imposes a configurational change on the enzyme proteins embedded in

the membranes. According to Lyons (1973), chill resistance develops by an increase in the unsaturated fatty acids of the lipids in the subcellular membranes. An increased unsaturation of the membrane lipids would cause the liquid crystalline state to be maintained at chilling temperatures. The phase transition theory has been verified for a number of chill sensitive plants, including *Gossypium hirsutum*, *Phaseolus vulgaris* (Wilson and Crawford 1974), and *Vigna radiata* (Raison and Chapman 1976). A number of herbaceous plants capable of developing freezing resistance have also shown an increase in the unsaturated fatty acids when exposed to low temperatures. The synthesis of unsaturated fatty acids and phospholipids was stimulated at low temperatures during hardening of *Triticum aestivum* (de la Roche et al. 1972, de la Roche, Pomeroy, and Andrews 1975), *Medicago media*, and *Medicago sativa* (Grenier et al. 1975). However, there was a stimulation in the synthesis of phospholipids without an increase in the unsaturated fatty acids in hardening *Robinia pseudoacacia* bark (Siminovitch, Singh, and de la Roche 1975). Singh, de la Roche, and Siminovitch (1975) suggested that the extreme frost resistance that develops in woody trees and shrubs is not merely due to an increase in the unsaturation of the fatty acids or a shift in the classes of phospholipids synthesized. Several years of study of the cold hardy *Robinia pseudoacacia* (Siminovitch et al. 1968, Siminovitch et al. 1975, Singh et al. 1975) and recent studies of cold

hardy _Pinus resinosa_ (Pomeroy, Siminovitch, and Wightman 1970) have culminated in the conclusion that the hardiness of woody trees and shrubs is developed by an augmentation of the whole protoplasm. Membrane replication, occurring by the increase in the polar lipid and phospholipid fractions, and cytoplasmic augmentation, indicated by an increase in the soluble sugars and proteins and nucleic acids leads to the total protoplasmic augmentation, and thus, cold hardiness.

Inhibitors of ribonucleic acid (RNA) and protein synthesis were found to disrupt the frost hardening process in the alga, _Chlorella ellipsoda_ (Hatano et al. 1976). Increases in the RNA fraction of hardening cells was found in _Robinia pseudoacacia_ bark (Siminovitch et al. 1968), _Cornus stolonifera_ bark (Chen and Li 1977) and apple twigs (Li and Weiser 1969). The increases in RNA were found prior to the increased protein synthesis that accompanied cold acclimation, and thus, supported the concept of protoplasmic augmentation in the development of cold hardiness.

Other hypotheses for the mechanism of freezing resistance have been proposed. Levitt (1972) proposed a sulfhydryl-disulfide hypothesis for cold hardiness. He suggested that freezing injury occurs as the protein molecules approach each other during freeze-dehydration. There is chemical combination between adjacent protein molecules and an oxidation of the sulfhydryl (SH) groups to form intermolecular disulfide (SS) bridges. The proteins undergo conformational changes upon freezing and thawing,

and injury occurs. Although Levitt (1972) recognized that the processes leading to protoplasmic augmentation occur during hardening, he believed these processes are primarily to prevent the intermolecular bonding between proteins.

Studies have shown that there is a strong interrelationship between drought and chill or cold hardening (eg. Levitt 1972). Wilson (1976) found that if Phaseolus vulgaris was exposed to dry atmospheric conditions prior to chilling temperatures, its chill resistance increased. Rikin and Richmond (1975) found that after tobacco and barley plants were grown under drought conditions, their cold hardiness increased. Chen, Li, and Weiser (1975) found that an imposed water stress increased the cold hardiness of Cornus stolonifera.

The water status of a plant is an integral factor in the cold hardiness of the tissue, yet very few studies have examined this aspect. It has been found that overwintering plant tissue becomes dehydrated. The water contents of Tsuga canadensis, Abies balsamifera, Picea rubens, and Thuja occidentalis needles were lowest in late winter (Clark and Gibbs 1957). Bannister (1964) studied the seasonal variation of the water content of Erica tetralix, Erica cinerea, and Calluna vulgaris leaves. He found that the three heath plants had the lowest seasonal water content from December to May. The water content of the stem cortex tissue of Cornus stolonifera was found to decrease with hardening (McKenzie, Weiser, and Li 1974b).

Measurements of total leaf water potential and its components are better indicators, than water content, of the physiological stresses being imposed on the tissue. Very few studies have measured the water potential of overwintering tissue. Lindsay (1971) found that _Abies lasiocarpa_ and _Picea engelmanni_ had annual minimum water potentials below -30 bars in the winter. Table 3 lists the seasonal changes in the osmotic potential of a number of overwintering plants. Although these studies of the seasonal variation of the water relations of cold hardy plants have been conducted, no studies have monitored the seasonal changes in the total leaf water potential and all its components and related them to tissue cold hardiness. Few attempts have been made to investigate the physiological significance (other than biochemical) of the freeze-dehydration events.

The present study was undertaken with a holistic viewpoint. The purpose of the study was to intensively study the seasonal variation in the leaf water relations of a broadleaf evergreen, _Ledum groenlandicum_, and relate the changes to the leaf cold hardiness. The majority of studies investigating the water relations and cold hardiness of a plant have dealt with either needles of conifers (eg. Parker 1963a) or the bark of deciduous plants (eg. McKenzie et al. 1974b).

The study was not intended to single out the leaf water status as the sole variable in determining the plants cold hardiness. Instead, it was hoped that by studying the leaf

Table 3. The minimum seasonal osmotic potential values for various cold hardy plants.

| Plant Species | Osmotic Potential bars | | Author |
|---|---|---|---|
| | Summer | Winter | |
| Picea engelmanni | -23.6 | -49.4 | Goldsmith and Smith 1926* |
| Chelidonium majus | -12.4 | -15.7 | Walter 1931* |
| Parietaria ramiflora | -14.8 | -20.9 | Walter 1931* |
| Hedra helix | -14.7 | -25.0 | Walter 1931* |
| Saxifraga caesia | -15.8 | -20.7 | Pisek and Cartellieri 1934* |
| Carex firma | -22.9 | -33.5 | Pisek and Cartellieri 1934* |
| Pinus cembra | -23.0 | -30.0 | Pisek, Sohm, and Cartellieri 1935 |
| Vaccinium myrtillus | -10.0 | -25.0 | Havas 1971 |
| Picea mariana | -15.6 | -56.0 | van Zinderen Bakker 1974** |
| Dryas integrifolia | -17.0 | -60.5 | Hartgerink 1975*** |

* data from Walter and Kreeb 1970.
** combined osmotic and matric potential
*** as for (**) under controlled environment.

water relations of a cold hardy evergreen plant, a better understanding of the total cold hardiness mechanism could be obtained. To accomplish these goals, the following areas were investigated.

A) Under field conditions:

1) the seasonal variation in the total leaf water potential and its components,

2) the seasonal variation in leaf tissue cold hardiness.

B) Under controlled environment conditions:

1) the effect of photoperiod and temperature on the cold acclimation and the water relations of _Ledum groenlandicum_,

2) the susceptibility cf the xylem sap of _Ledum groenlandicum_ to cavitation,

3) the changes in the Höfler relationships of hardened versus unhardened _Ledum groenlandicum_ leaf tissue,

4) the effect of tissue hardiness on transpiration rates,

5) the effect of tissue hardiness on net photosynthetic and dark respiration rates.

The results of this study will hopefully further the knowledge of the cold hardiness mechanism and the ecophysiology of members of the Ericaceae and augment the knowledge of the cold hardiness mechanisms of other plants.

7

## MATERIALS AND METHODS

### Plant Materials

_Ledum groenlandicum_ Oeder plants used in the controlled environment studies were collected from the periphery of a black spruce/ labrador tea bog near Whitecourt, Alberta in September 1975 and June 1976. The soil from the collection site was retained as the potting medium. The plants were transferred to a greenhouse and were grown under a 15 to 20 C temperature regime, a relative humidity of 50%, and a 16 h photoperiod (natural daylength extended with Multivapour and Lucalox lamps, giving an illuminance of 1500 ft-c (photosynthetically active radiation (PAR): 270 $\mu E$ $m^{-2}$ $s^{-1}$) at the growing bed level). Plants were watered every second day with distilled water. After growing at least one month in the greenhouse, the plants were transferred to controlled environment chambers (Environmental Growth Chambers, Chagrin Falls, Ohio) for specific studies.

General Methods and Materials


## Cold Hardiness Determinations


Cold hardiness was determined by subjecting three, 8 to
10 cm leafy shoots per temperature treatment to a controlled
freezing stress as described by McKenzie and Weiser (1975).
For each temperature treatment, the cut ends of the shoots
were consecutively wrapped with a 2 cm wide strip of damp
cheesecloth and secured with cotton thread. The shoots were
taped to aluminum foil and a .076 mm Cu-constantan
thermocouple (see Appendix B, p. 136) was attached to a
leaf. The shoots were wrapped in the aluminum foil, and with
the cheesecloth strip and thermocouple wire extended, the
unit was placed in a thermos flask that was precooled to
5 C. The thermos was sealed with Terostat VII (Terosan,
Heidelberg) and placed in a freezing cabinet set at -15 C.
The ambient temperature was lowered in steps of 10 C every 2
h to -30 C. To reach temperatures below the freezer's
cooling capacity, a thermos was mounted on a Peltier cooling
plate (Thermoelectrics Model TCP-2) inside the freezer.
Using this procedure, shoots could be cooled at 3 to 5 C h$^{-1}$
to -40 C. When a treatment reached the desired temperature
the thermos was removed from the freezer and thawed in a 5 C
chamber for 24 h.

Visual Rating of Injury

Thawed, cold-stressed shoots were placed in sand on a misted propagation bench in a 21 to 26 C greenhouse. After 2 weeks, tissue viability was rated according to leaf colour, midrib vein colour, and terminal bud condition (see Table 4) After one month, growth was assessed. Shoots were considered alive if root growth or bud break had occurred, or if, in the absence of growth, the leaves were green and the midrib veins were white. The temperature treatment corresponding to 50% survival was designated as the survival temperature (also known as LT-50). The visual rating was used as a direct measurement of injury and as a control for evaluating the triphenyl tetrazolium chloride test.

Triphenyl Tetrazolium Chloride Test

The triphenyl tetrazolium chloride (TTC) test for tissue viability was adapted from Steponkus and Lanphear (1967a). Three, 50 mg samples made up of approximately 0.25 cm$^2$ leaf sections were taken from the leaves of each temperature treatment. The leaf tissue was placed in 3 ml of 0.6% (w/v) TTC in 0.05 M $Na_2HPO_4$-$KH_2PO_4$ buffer (pH 7.4) + 0.05% (v/v) wetting agent (Triton-X), infiltrated under vacuum, and incubated at 30 C for 15 h. The TTC solution was drained and the leaf tissue was rinsed with distilled water. The red formazan derivative was extracted from the leaf

Table 4.  Rating of visual injury of cut shoots of Ledum groenlandicum placed in sand following controlled freezing tests.

| Time | Survival Observations | | |
|---|---|---|---|
| weeks | | min | max |
| 2 | Browning | | |
| | leaf colour | (0)-brown | (4)-green |
| | midrib vein colour | (-)-brown | (+)-white |
| | Bud damage | (-)-open | (+)-compact |
| 4 | Growth | | |
| | roots | (-)-absent | (+)-present |
| | bud break | (-)-absent | (+)-present |

tissue with 7 ml of 95% (v/v) ethanol in a boiling water
bath for 5 min. The extracts were co... ...d and made up to 10
ml with 95% ethanol. The absorbance was recorded at 530 mµ
on a Beckman DB-G spectrophotometer (Beckman Instruments
Inc., Fullerton, Calif.) and was expressed as a percentage
of the absorb... of the control temperature treatment
(5 C). The survival temperature was derived from the
interpolation of the 50% absorbancy value (see Fig. 1). A
good correlation existed between the 50% survival of the
shoots after 4 weeks in moist sand and the 50% absorbance
value obtained from the TTC test (Fig. 2).


Water Relations


Thermocouple Pshychrometers


Total leaf water potential ($\psi$) and the component
potentials ( $\psi p$ and $\psi_\pi + _\tau$, eq. 1) were measured with
Spanner-type thermocouple psychrometers, constructed after
Mayo (1974), and a Fluke model 845 AB high impedance
voltmeter. A typical calibration curve is given in Appendix
A, p. 131. To determine $\psi$, three 4 mm diameter leaf discs
were placed in a 4 mm deep by 4 mm in diameter sample
chamber. Three readings, at 5 min intervals, were taken
after a 4 h equilibration period in a 30 C constant
temperature bath. Replicate readings were within 0.2 uV or
0.3 bars agreement. Longer equilibration times (i.e. 12 to
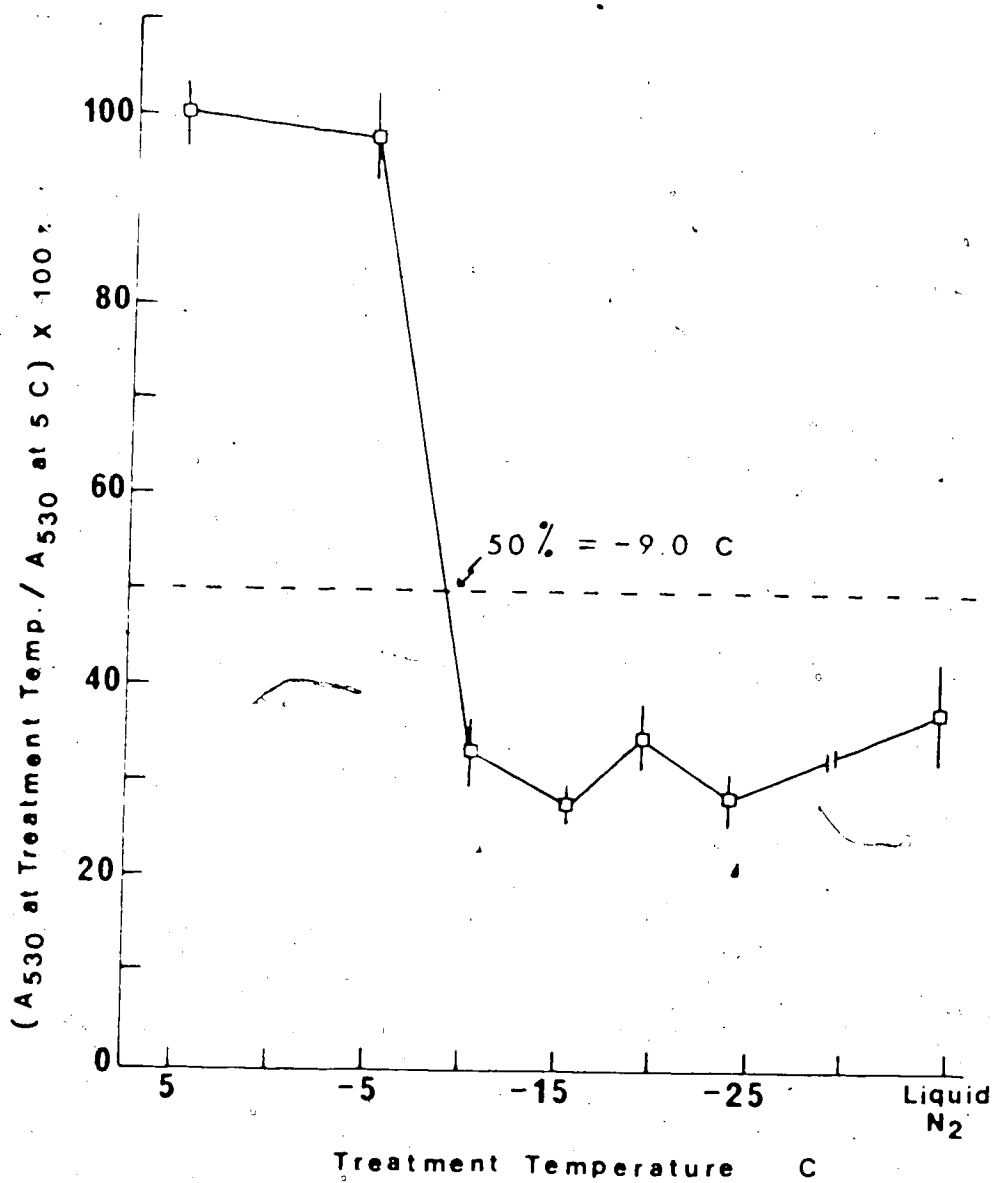
Fig. 1. The effect of low temperature stress on Ledum groenlandicum leaf tissue viability as determined with triphenyl tetrazolium chloride. Each symbol represents the mean of three samples. Vertical bars indicate the standard error of the mean. The tissue survival temperature was found by the interpolation of the 50% absorbancy value. The above sample was from August 20, 1976.
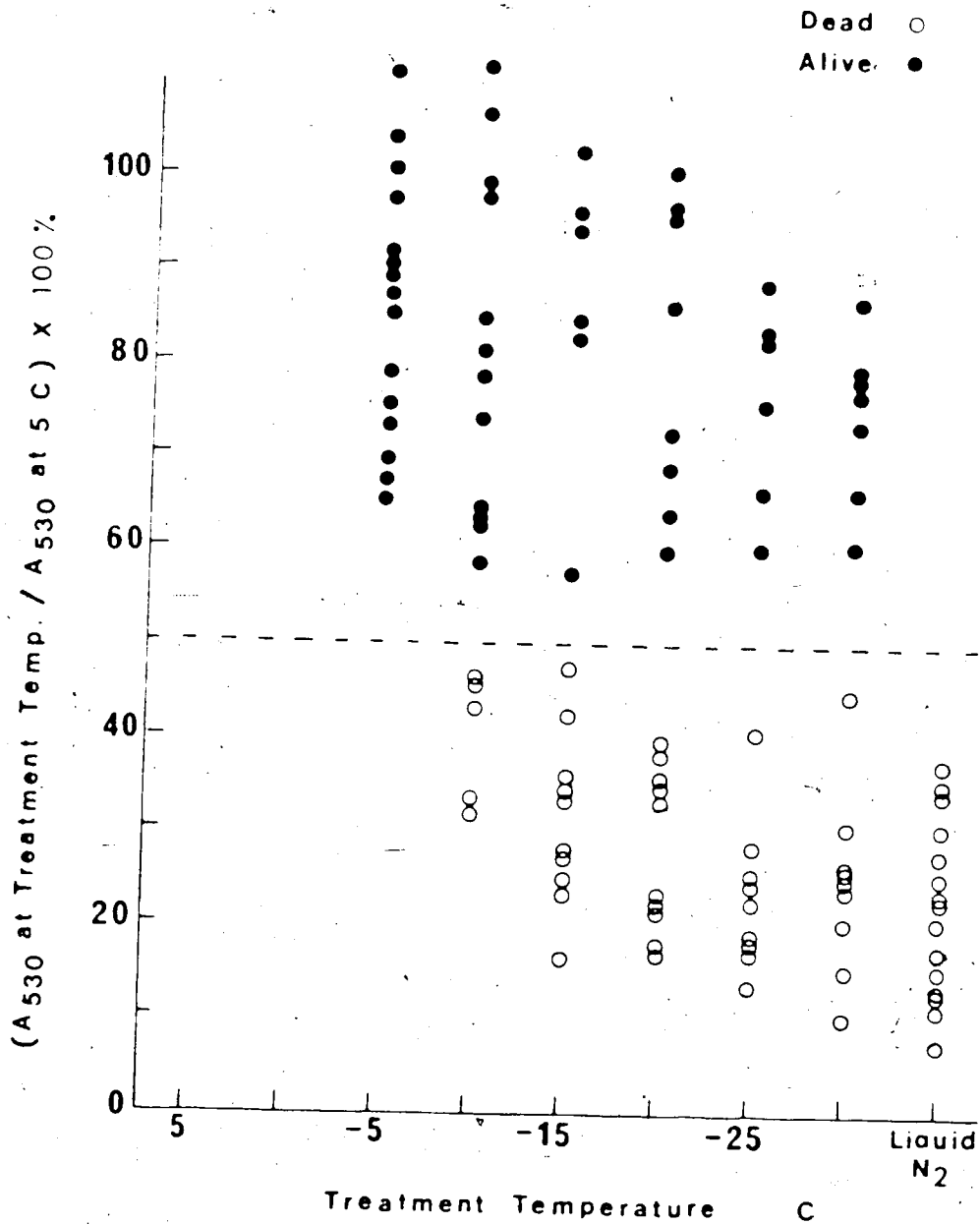
Fig. 2.  The post-freezing survival of <u>Ledum groenlandicum</u> shoots after 4 weeks in moist sand as correlated with the percent reduction of triphenyl tetrazolium chloride.

24 h) caused the tissue to deteriorate.

To determine the component potential, $\Psi_{\pi}+_{\tau}$, the sample chamber was wrapped in aluminum foil and immersed in liquid $N_2$ (-196 C) for 10 min to rupture cell membranes. The chamber was then warmed for 40 min and returned to the thermocouple psychrometer. After a 1.5 h equilibration period readings were taken as for .

Turgor pressure ($\Psi p$) was derived using the equation:

$$\Psi p = \Psi - \Psi_{\pi}+_{\tau}$$  (1)

where
$\Psi p$ = turgor pressure,

$\Psi$ = total leaf water potential, and

$\Psi_{\pi+\tau}$ = combined osmotic and matric potential.

Scholander Bomb

Xylem tension was measured with a Scholander Bomb (PMS Instrument Co., Corvalis, Oregon). Leafy shoots, 7 to 10 cm long, were placed in the chamber and the pressure was raised at about 1 bar per 5 s. Readings were recorded when the xylem sap was seen, with the aid of a handlens, to return to the cut surface of the stem.

Relative Water Content

Relative water content (RWC) is defined as (eg. Barrs

1968):

$$RWC = \frac{fresh\ wt - dry\ wt}{turgid\ wt - dry\ wt} \qquad (2)$$

Three, 7.5 mm in diameter leaf discs comprised each weighing sample. All weights were measured with a Mettler H10 balance (Zurich, Switzerland). The fresh wt was recorded immediately after the discs were cut.

For the determination of the turgid wt the individual leaf discs were placed in a saturation chamber, similar to that described by Slavik (1974), consisting of a wet foam sheet in a petri dish (see Appendix A, p.131). The cut edge of each leaf disc made firm contact with the wall of the saturated foam hole, and full turgidity was reached after 3 h saturation in the dark at 22 C (see Appendix A, p. 131). The turgid leaf discs were removed from the saturation chamber, blotted dry between several layers of filter paper for 10 s and weighed. The sample was then oven-dried at 55 C for 48 h and the dry wt recorded.

Potometers

Simple potometers were constructed to measure the rate of water uptake by cut shoots. The potometer consisted of a 0.2 ml pipette calibrated to 0.001 ml and a 5 ml pipette that served as a reservoir and was sealed from the water uptake column by a clamp. A short rubber tube connected the pipette system to the shoot assembly.

The cut shoot was recut and threaded through the rubber
stopper under water. The shoot-stopper assembly was
connected to the water filled potometer system and
five-minute epoxy was applied to seal the
tubing-stopper-shoot connections. The open ends of the
potometer were covered with aluminum foil to prevent
evaporation (see Appendix A, p. 131).

Gas Exchange

Gas Analysis System

Net photosynthetic rates were determined using
infra-red gas analysis in an open system (Šesták, Čatský,
and Jarvis 1971). The output from a Unor II differential
analyser (Maihak, Hamburg), spanned at 35 ppm full scale,
was continuously recorded on channels 1 to 8 of a Honeywell
Electronic 16 dual range, 24 channel, strip chart recorder.
Channels 9 to 24 were calibrated over a range of -10 to
+40 C for Cu-constantan thermocouples. The system design was
after Mauer (1977), but had modifications for increased flow
rates. The flow rates of the reference and sample gases
through the analyser were monitored with Gilmont size 2
flowmeters. Constant flow rates through the system were held
between 330 ml min$^{-1}$ and 530 ml min$^{-1}$, depending on the
plant size.

Single leafy branches were sealed within a cuvette

equipped with an internal fan. The design of the gas flow system and the cuvette are given in Appendix B, p. 136.

On each day of measurement, the infra red gas analyser (IRGA) was calibrated with standard gases so that the chart recordings in mV could be converted directly to ppm $CO_2$. The standard gases were calibrated against gases of known concentration with Wosthoff pumps as described by Bate, D'Aoust, and Canvin (1969). The differential concentration of $CO_2$ between the ambient air stream and the sample air stream was determined by alternating between a 10 min interval of an ambient/ambient air stream and a 20 min interval of a cuvette/ambient air stream. The chart recordings were visually averaged over each interval, and the interval values obtained were averaged over the period of measurement at a particular regime.

## Environmental Measurements

The temperature of the cuvette was controlled by adjusting the temperature of the entire growth chamber. Leaf and air temperatures inside the cuvette were monitored at midshoot height with .127 mm Cu-constantan thermocouples (Omega Engineering, Stamford, Conn.) (see Appendix B, p. 136). Leaf thermocouples were placed on the abaxial leaf surface. Thermocouple outputs were recorded continuously with a Honeywell multipoint strip chart recorder.

Light was provided by the standard growth chamber

lighting fixture supplemented with a quartz iodide lamp (GTE Sylvania, Drummondville, Que.). The lamp was suspended above the cuvette and the light was filtered through 5 cm of water. The light intensity generated by the lamp was adjustable with a variable transformer. Photosynthetically active radiation (PAR: 400 to 700 nm) was measured at midshoot height outside the cuvette with a Model LI-185 meter (Lambda Instruments, Lincoln, Nebraska). Spectral analyses of the light sources are given in Appendix C, p. 140. No humidity control was attempted, nor was the humidity of the sample air stream monitored during measurements.

Calculation of Net Photosynthesis

After completion of the $CO_2$ gas exchange measurements, the area of the attached leaves was estimated after the method of Ackley, Crandell, and Russel (1958). The correlation coefficient and the linear regression equation used to estimate leaf area from the product of the leaf length and width are given in Appendix D, p. 142. Leaf areas were measured by tracing the leaf outline on graph paper and counting 1 mm$^2$ squares. Leaf length was measured from the tip of the leaf blade to the petiole. Leaf width was measured at the widest point of the leaf, perpendicular to the length axis.

Rates of net photosynthesis and dark respiration were calculated as mg $CO_2$ dm$^{-2}$ h$^{-1}$ using the following equation

adapted from Hartgerink (1975):

Net photosynthesis of $CO_2$ (mg $dm^{-2}$ $h^{-1}$) =                    (3)

$$(C \text{ ppm} * Y \frac{mg}{ml \ ppm} * \frac{273 \ K}{Ta \ K} * F \frac{ml}{min} * 60 \frac{min}{h}) : A \ dm^2$$

where

    C = $CO_2$ assimilated in ppm

    Y = conversion from ppm to mg $ml^{-1}$

      = $\frac{44 * 10^{-6}}{22.414} \frac{mg}{ml \ ppm}$ (Šesták, Čatský, and Jarvis 1971)

   Ta = air temperature in K

    F = flow of air through cuvette in ml $min^{-1}$

    A = lower leaf surface in $dm^2$

A linear regression equation for leaf dry wt as a function of leaf area was determined on a small sample (see Appendix D, p. 142), and thus, the calculated net photosynthetic rates can be converted to mg $CO_2$ dry wt $h^{-1}$.

Field Studies

Site Description

The Ledum groenlandicum plants used in the field study were growing at the University of Alberta Devonian Botanic Gardens, 25 km SW of Edmonton. The population of Ledum groenlandicum selected for sampling was situated west of the Shrubs and Spring Bulbs plot. The plants were 20 to 60 cm

high and were growing in a depressed area, under a _Populus_
_tremuloides_ canopy, south of a small man-made pond.

### Sampling Schedule

The water relations and cold hardiness of _Ledum_
_groenlandicum_ were monitored monthly or biweekly from
November 1975 to April 1977. Cut shoot samples were taken at
sunrise and at noon and transported about 100 m in a closed
humid chamber to a hut. Leaf water potential and its
components were measured with thermocouple psychrometers.
The leaf discs were loaded into the thermocouple
psychrometer chambers in the hut and then the water
potential readings were taken after an equilibration period
in the Edmonton laboratory. Xylem tensions were determined
with a Scholander Bomb immediately after the psychrometer
chambers were loaded. During the winter, shoots were allowed
to thaw 15 min before xylem tension determinations were
attempted. The cut shoots were transported in the sealed
humid chamber to the Edmonton laboratory for cold hardiness
determinations.

Controlled Environment Studies

Photoperiod Effect on Cold Acclimation and Water
Relations

To assess the importance of photoperiod in the
induction of cold hardiness, Ledum groenlandicum plants were
grown under either short days (8 h) or long days (16 h).
Eight plants, 20 to 40 cm high, were subjected to each
controlled environment regime.

The illumination was provided by 20 Sylvania F48T12
CW-VHO or Phillips TL 110W 33RS fluorescent lamps and 6 100W
incandescent lamps. In both chambers, the light intensity at
the growing bed level was 440 $\mu E\ m^{-2}\ s^{-1}$ (PAR) (27,100 lux).
Spectral analysis of the chamber lighting is given in
Appendix C, p.140. Light intensities were measured as
described on p. 35.

The two photoperiod treatments were subjected to
identical temperature regimes. The temperature regimes were:
25/15 C day/night for 4 weeks, followed by 15/5 C day/night
for 2 weeks, and ending with 15/5 C day/night with 2 h of
-2 C night frost for 2 weeks. Relative humidity, determined
by the chamber dew point depression, was maintained at 70%.
Prior to the experiment, the plants had terminated a period
of rapid growth. The plants were well- watered throughout
the experiment.

The survival temperature (p. 26), the total leaf water

potential and its components (p. 29), and relative water content (p. 32) were measured following the schedule given in Table 5.

## Cavitation in Frozen Stems

Methods similar to those of Hammel (1967) were used to determine the degree of cavitation after freezing in the stems of _Ledum groenlandicum_ and _Pinus banksiana_.

### Water Uptake and Weight Loss

In the first two cavitation studies on _Ledum groenlandicum_, both the water uptake and weight loss of the cut shoots were measured and found to be the same, therefore, in the remainder of the cavitation studies only water uptake was recorded. The water uptake and weight loss of the cut snoots were measured as a function of time. Cut shoots were weighed and mounted into potometer systems (p. 33.) If weight loss was recorded, the potometer system was mounted onto a Mettler P1200 balance accurate to 0.01 g. Studies were conducted in a growth chamber having a light intensity of 161 $\mu E$ $m^{-2}$ $s^{-1}$ (PAR) (9,720 lux) at the shoot height, 27 C air temperature, and a relative humidity of 56%. A plastic shielding around the potometer-balance assembly minimized the air flow while readings were being made.

Table 5.   The controlled environment regime and sampling schedule used in the study of the effect of photoperiod on the cold acclimation and the water relations of <u>Ledum groenlandicum</u>.

| Sampling Time | PAR* $\mu E\ m^{-2}\ s^{-1}$ | | Air Temperature C | | |
|---|---|---|---|---|---|
| weeks into study | time | | time at max | | |
| | h | intensity | h | max | min |
| 0 | 16 | 270 | 24 | 15-20 | - |
| 4 | 8 | 440 | 16 | 25 | 15 |
| | 16 | 440 | 16 | 25 | 15 |
| 6 | 8 | 440 | 16 | 15 | 5 |
| | 16 | 440 | 16 | 15 | 5 |
| 8 | 8 | 440 | 16 | 15 | -2 |
| | 16 | 440 | 16 | 15 | -2 |

*PAR=photosynthetically active radiation.

The potometer system was equilibrated for 1 h, then readings were made at 15 min intervals for 2 h prior to the stem being frozen. To freeze the stem, a 2.5 cm d rubber sleeve was attached to the stem and liquid $N_2$ was pipetted into the sleeve for 15 min. During freezing, the plant foliage and potometer were protected with aluminum foil from the liquid $N_2$. The rubber sleeve and aluminum foil were removed after the freezing period and readings were resumed for 2 h at 15 min intervals, and then at 30 min intervals for the remainder of the light period. Recovery rates were monitored at 24 h after the freezing.

## Xylem Tension

The effect of freezing on the xylem tension of a branch of an intact plant was determined. Stems were frozen in the late morning or early afternoon when xylem tensions were high. The xylem tension of a neighbouring (pre-freeze control) shoot was measured with a Scholander Bomb immediately prior to the freezing of the experimental branch. A 2.5 cm deep rubber sleeve was secured to a stem section leading to two shoots of the experimental branch. The branch foliage was shielded with aluminum foil and the stem was frozen for 10 min with liquid $N_2$.

The freezing apparatus was dismantled and after 7 h the xylem tensions of an unfrozen shoot (post-freeze control) and of one the shoots of the branch that had been frozen for

10 min (post-freeze experimental) were measured. The remaining intact frozen shoot was observed over a 4 week period for signs of damage.

### Physiological Responses of Hardened versus Unhardened Tissue

Environmental Treatments

The effects of photoperiod and temperature on the cold acclimation of Ledum groenlandicum were determined in preliminary studies (p. 39). Based on these results, plants were subjected to a hardening regime (see Table 6) so that a comparison of selected physiological processes in hardened and unhardened plants could be made. 'Unhardened' refers to plants grown under the 16 h day, 25/15 C temperature regime. The same plants were subjected to the hardening regime, and 'hardened' refers to plants that had grown at least 2 weeks under the 8 h day, 15/5/-2 C temperature regime. An exception to the above hardening regime is that of the hardened plants in the transpiration study. 'Hardened' material refers to those plants that were grown under the 8 h photoperiod regimes described on p. 39.

Höfler Diagrams

Höfler diagrams (as described by Slatyer 1967) were

Table 6. The controlled environment regime used in the studies of the physiological responses of hardened vs. unhardened Ledum groenlandicum. Plants light intensity was measured at the growing bed level. Plants were between 20 and 40 cm high.

| Simulated Season | Season Length (Weeks) (h) | PAR µE m⁻² s⁻¹ intensity | Air Temperature C h | Air Temperature C max | Air Temperature C min | Comments |
|---|---|---|---|---|---|---|
| late summer | 4   16 | 280 | 16/8 | 25 | 15 | *LT-50 = -9 C terminal buds |
| early fall | 4   8 | 140 | 8/16 | 15 | 5 | red pigments in leaves |
| late fall | 2   8 | 50 | 8/16 | 15 | -2 | LT-50 = <-40 C no watering |

*LT-50 = survival temperature

constructed from water potential and relative water content
data obtained from the leaves of hardened and unhardened
plants.

Excised leaves were placed in a saturated chamber for
3 h, then the leaves were air dried for 0 to 1.5 h to obtain
varying levels of tissue hydration. Three leaves comprised
one corresponding water potential and relative water content
determination. From each leaf, one disc was removed for the
water potential measurement, and one disc was removed for
the relative water content determination.

The Höfler diagrams were analysed with the models of
Warren Wilson (1967a, 1967b, 1967c) and Acock (1975). Table
7 lists the calculated water relations constants and
variables and their equations.

## Transpiration Rates

Transpiration rates of hardened and unhardened plants
were measured with potometers (p. 33). Studies were
conducted in a growth chamber having a 220 $\mu E$ $m^{-2}$ $s^{-1}$ (PAR)
(17,250 lux) light intensity at the shoot height, a 20 C air
temperature, and a relative humidity of 50%. Leaf and air
temperatures were monitored with ~127 um Cu-constantan wire
thermocouples.

The cut shoots were transferred immediately from the
different growing regimes and were assembled into the
potometers. The potometer assembly was equilibrated for 1 h

Table 7. The derived water relations constants and variables used in the analysis of the Hofler diagrams.

| Derived Variable | Symbol | Equation* | Source |
|---|---|---|---|
| Osmotic potential | $\pi$ | $= (\pi_t + \tau_t)\dfrac{(1-B)}{R}$ | Warren Wilson 1967a |
| Matric potential | $\tau$ | $= (\pi_t + \tau_t)\dfrac{B(1-B)}{R(R-B)}$ | Warren Wilson 1967a |
| Bound water content | $B$ | $= \dfrac{R(\pi+\tau) - (\pi_t + \tau_t)}{(\pi+\tau) - (\tau_t + \tau_t t)}$ | Warren Wilson 1967c |
| Coefficient of enlargement | $e$ | $= (\pi_t + \tau_t) + \dfrac{(\pi+\tau) - (\pi_t+\tau_t) - \psi}{1-R}\cdot t$ | Warren Wilson 1967c |
| Combined osmotic and matric potential at full turgor | $\pi_t + \tau_t$ | $=$ extrapolation of plot of R vs $1/\pi+\tau$ | Acock 1975 |
| Intracellular combined osmotic and matric potential | $\pi_c + \tau_c$ | $= \dfrac{(R-B)((\pi+\tau)-\psi)) + \tau}{V_c}$ | Acock 1975 |
| Intracellular turgor pressure | $P_c$ | $= \psi - (\pi_c + \tau_c)$ | Acock 1975 |

*R=RWC, $\pi+\tau=\psi_{\pi+\tau}$, P=$\psi_p$.

before readings were taken at 1 h intervals. The influence of different light conditions on the transpiration rate was determined by subjecting the cut shoots to alternate 4 h periods of light and dark over a 12 h period.
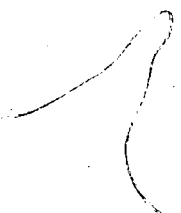
Net Photosynthetic Rates

The net photosynthetic rates of hardened and unhardened plants were measured with an IRGA system (p. 34). Plants were transferred immediately from the growing regime to the growth chamber equipped with the IRGA. Plants were well watered throughout the net photosynthesis study. An intact branch was mounted into the cuvette and the IRGA system was equilibrated for 1 h before measurements commenced.

The measurements of the net assimilation response to temperature, at 5 C intervals, were made over a range of 0 to 30 C leaf temperatures. The IRGA system was equilibrated at each temperature regime for 1 h before 3 h periods of measurement were recorded. Light intensity was maintained at 800 $\mu E$ $m^{-2}$ $s^{-1}$ (PAR) during the net photosynthesis determinations. Temperature regimes were progressively changed from the warmest to the coldest regime. After all the temperature regimes in the light were completed the response of dark respiration to temperature was determined. The measurement period was 2 h. The maximum time a shoot was sealed in a cuvette was 72 h.

Light intensity response measurements were made over

the range of 0 to 1600 $\mu E$ $m^{-2}$ $s^{-1}$ (PAR), at 25 C for unhardened plants and 15 C for hardened plants; these temperatures gave the optimal photosynthetic rates. After 30 min equilibration at each light intensity, measurements were taken for 2 h periods. The light intensity was varied from the highest intensity, stepwise to darkness.

EXPERIMENTAL RESULTS


Field Studies


Seasonal Variation in Cold Hardiness


The cold hardiness (or survival temperature) of <u>Ledum</u>
<u>groenlandicum</u> plants growing at the University of Alberta
Devonian Botanic Gardens was monitored (as described on p.
38) from February 22, 1976 to April 24, 1977.

Weekly summaries of meteorological data from November
1975 to April 1977, for the Edmonton International Airport,
12 km SE of the University of Alberta Devonian Botanic
Gardens, are given in Fig. 3. Maximum temperatures were
below 10 C from mid-November 1975 to the end of March 1976.
In early April 1976 maximum temperatures rapidly rose and
remained above 20 C until the first wee: in October 1976.
During late November 1976, maximum temperatures decreased
and remained below 10 C until April 1977. Minimum
temperatures were below freezing from November 1975 to mid
May 1976 and from the first week in October 1976 to the end
of April 1977. The annual cycle of the total possible hours
of sunshine had maximum and minimum values of 17.1 h and 7.5
h respectively. Daylength was longer than 13 h by early
April and shorter than 13 h by early September. Snow covered
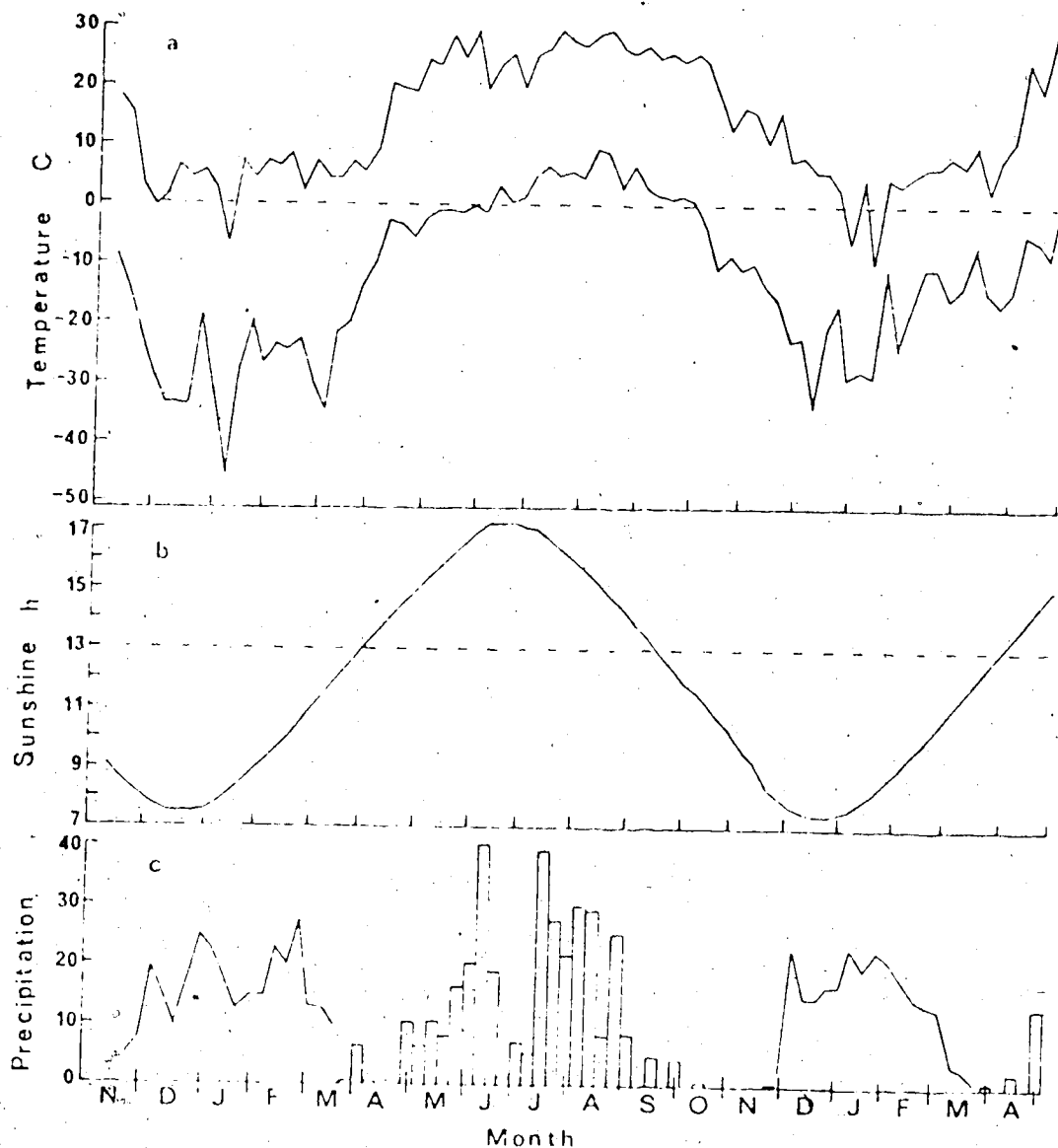
Fig. 3. Weekly summaries from November 1975 to April 1977 of a) maximum and minimum air temperatures, b) total possible hours of bright sunshine, and c) ground snow cover (cm) (line graph) and total rainfall (mm) (bar graph) for the Edmonton International Airport.

the ground from November 1975 to mid-March 1976, and from the end of November 1976 to late March 1977. Precipitation was generally greater than 10 cm per week from the beginning of May 1976 to mid September 1976.

The seasonal variation in the cold hardiness of Ledum groenlandicum (see Fig. 4) can be related to the changing meteorological environment. The survival temperature of Ledum groenlandicum was below -30 C (the minimum measurable temperature) from February 22, 1976 to April 18, 1976. A rapid rise in the survival temperature to -12.5 C occurred between April 18, 1976 and May 23, 1976. During this period the maximum temperature had risen above 20 C, daylength was longer than 13 h, the ground snow cover had completely melted, and bud break had occurred. Throughout the summer months, when minimum temperatures were above freezing, the survival temperature of Ledum groenlandicum was about -9 C. By September 26, 1976 the daylength was shorter than 13 h, but minimum temperatures were still above freezing. Terminal buds had formed on the shoots of Ledum groenlandicum and the survival temperature had decreased to -21 C. With subfreezing temperatures by October 10, 1976, the red leaf colouration (possibly anthocyanins) became more apparent and the survival temperature rapidly decreased. By November 20, 1976 the survival temperature was below -40 C (the minimum measurable temperature). The survival temperature did not increase until April 24, 1977, after a 3 week period of maximum temperatures above 20 C and daylength longer than
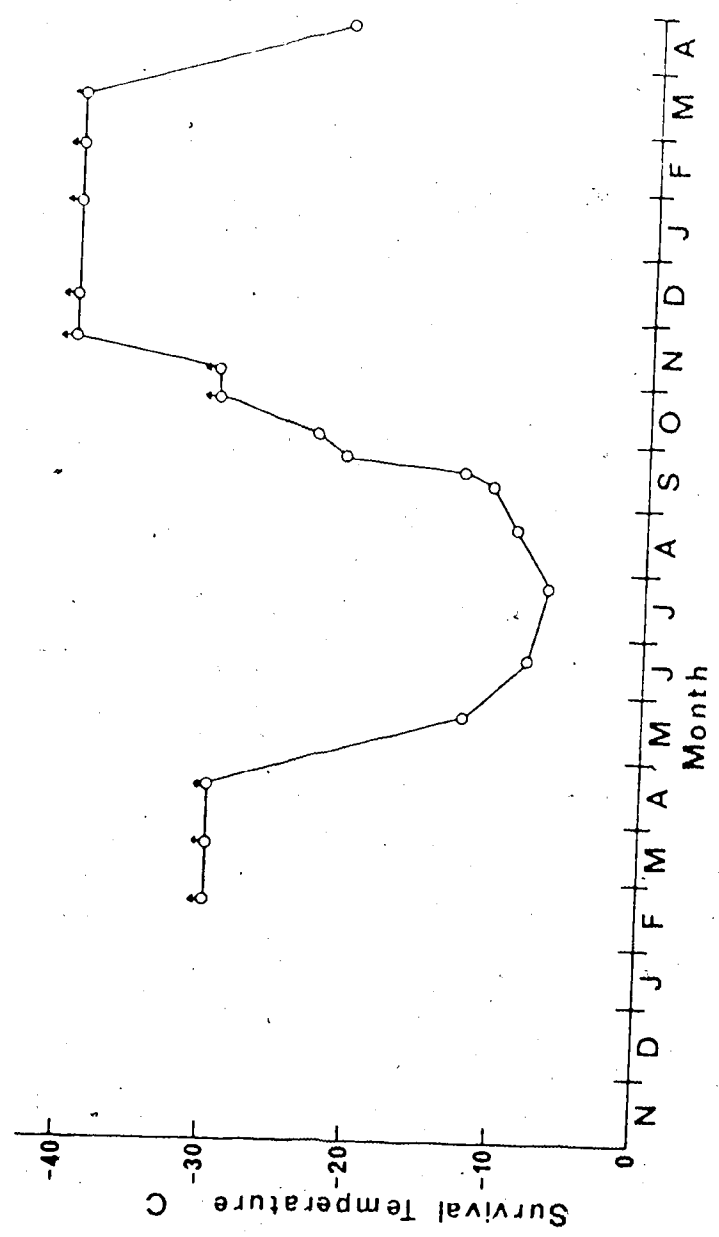
Fig. 4. The seasonal variation of the survival temperature from February 1976 to April 1977 of <u>Ledum groenlandicum</u> growing at the University of Alberta Devonian Botanic Gardens. Arrows indicate a survival temperature that was below the coldest obtainable freezing temperature. Prior to November 1976, the cooling capacity of the freezer was -30 C. A modification of the freezing method in November 1976 increased the freezer's cooling capacity to -40 C.

13 h. Bud break still had not occurred.


Seasonal Variation of the Water Relations


The seasonal variation of the water relations of Ledum groenlandicum, measured over the period from November 8, 1975 to April 24, 1977, can be related to the environmental factors given in Fig. 3.

Total leaf water potential (see Fig. 5) and xylem tension (see Fig. 6) followed similar patterns. Between December 1975 and March 21, 1976, the water potential was below bars and the xylem tension was greater than 40 bars. Readings below the lower limit of the thermocouple psychrometer and above the upper limit of the Scholander Bomb (-67.5 bars and 65 bars respectively) were common. With snow melt and warmer maximum temperatures, from March 21, 1976 to April 18, 1976, water potential increased. Between April 18, 1976 and November 6, 1976 the total leaf water potential was above -25 bars. The xylem tension showed a diurnal variation throughout this period. After November 6, 1976, when minimum air temperatures were below -15 C, the leaf water potential and xylem tension dropped below -67.5 bars and increased above 65 bars respectively. Values indicating severe water stress were recorded until March 21, 1977. By April 24, 1977, when the snow had melted and maximum temperatures were above 20 C, the water potential had risen above -25 bars and the xylem tension was below 25
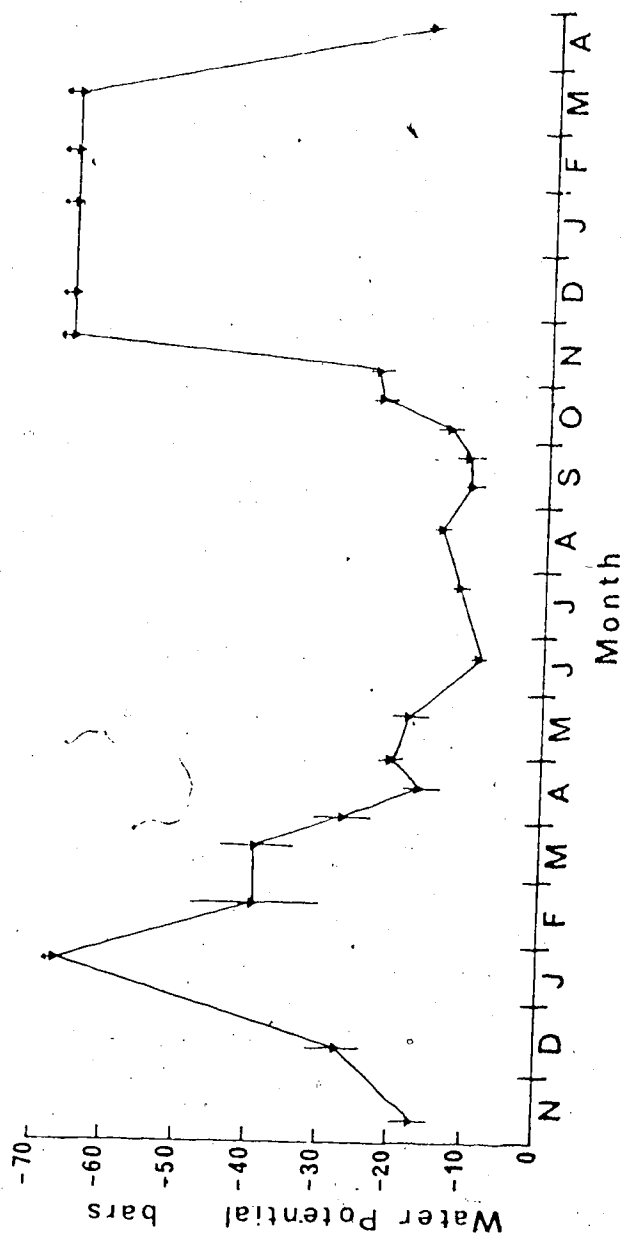
Fig. 5. The seasonal variation of the total leaf water potential from November 1975 to April 1977 of Ledum groenlandicum growing at the University of Alberta Devonian Botanic Gardens. Data points represent pooled dawn and noon psychrometer readings. Arrows indicate offscale readings. Symbols represent the mean of six samples. Vertical bars indicate the standard error of the mean.
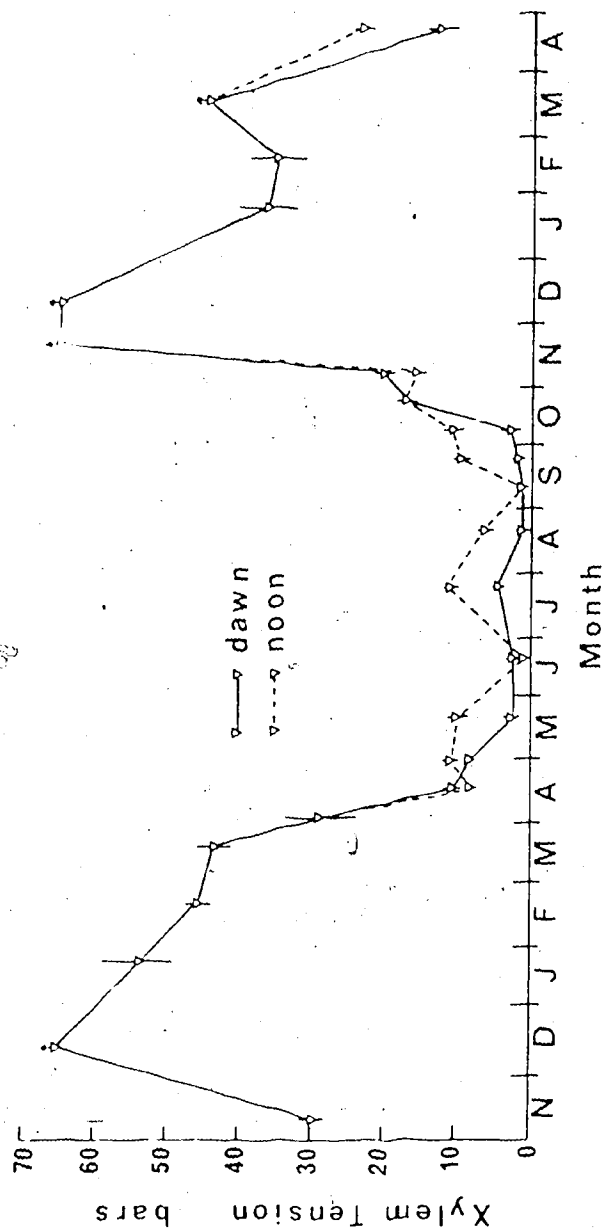
Fig. 6. The seasonal variation of the xylem tension from November 1975 to April 1977 of _Ledum groenlandicum_ growing at the University of Alberta Devonian Botanic Gardens. Diurnal variation is apparent in summer readings. Arrows indicate offscale readings. Symbols represent the mean of ten readings. Vertical bars indicate the standard error of the mean.

bars.

The combined osmotic and matric potential (Fig. 7) followed a similar seasonal trend as the total leaf water potential. Values were below -35 bars from November 6, 1975 to March 26, 1976 and from November 20, 1976 to March 20, 1977. Between April 18, 1976 and November 6, 1976 and by April 24, 1977, the combined osmotic and matric potential was above -30 bars.

Turgor pressure (Fig. 7) was below 3 bars from December 15, 1975 to March 21, 1976 (except February 23, 1976) and from October 23, 1976 to April 24, 1977. Negative turgor pressures were calculated several times throughout the winter. With snow melt and high maximum temperatures, turgor pressure had increased to a maximum of 9.1 bars by April 18, 1976. This was prior to bud break. Turgor pressure decreased and remained at 4.5 bars from May 24, 1976 to September 25, 1976.

## Controlled Environment Studies

### Photoperiod Effect

#### The Effect of Daylength on the Initiation of Cold Acclimation

The effect of daylength on the cold acclimation of Ledum groenlandicum was studied by subjecting two groups of
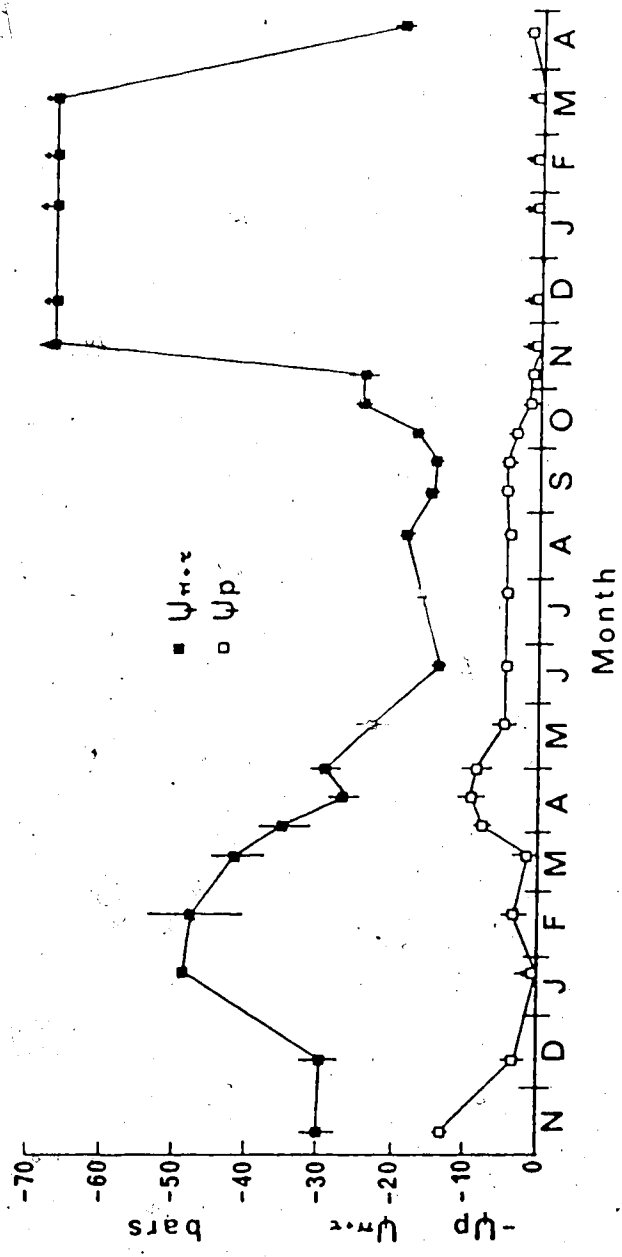
Fig. 7. The seasonal variation of the combined osmotic and matric potential (■) and the turgor pressure (□) from November 1975 to April 1977 of Ledum groenlandicum growing at the University of Alberta Devonian Botanic Gardens. Data points represent pooled dawn and noon psychrometer readings. Arrows indicate offscale readings (■) or calculated negative turgor pressures (□). Symbols represent the mean of six samples. Vertical bars indicate the standard error of the mean.

plants to identical temperature regimes, but different photoperiods; either 8 h (SD) or 16 h (LD) (see Fig. 8).

After 4 weeks under a warm 25/15 C day/night temperature regime, the survival temperature of plants grown under the LD photoperiod had decreased from -9 C to -12 C. After growing an additional 2 weeks at the 15/5 C day/night temperature regime, the survival temperature of the LD plants was -12.5 C. The survival temperature of the LD plants had decreased to -16.0 C after the final 2 weeks of growing at the 15/5/-2 C temperature regime.

Short day plants growing under the 25/15 C temperature regime for 4 weeks decreased in survival temperature from -9 C to -19.5 C. After 2 weeks under the 15/5 C temperature regime, the survival temperature of SD plants decreased further to -22.5 C. A rapid fall in survival temperature to below -30 C (the minimum measurable temperature) occurred after the final 2 week 15/5/-2 C temperature regime.

The Effect of Daylength on the Tissue Water Balance

The water relations of the plants subjected to the SD and LD photoperiods were studied at the same sampling intervals (see Table 5, p. 41) as the survival temperature determinations in the preceding section. Plants were well-watered throughout the study.

The total leaf water potential of the plants grown under the LD photoperiod decreased from an initial mean
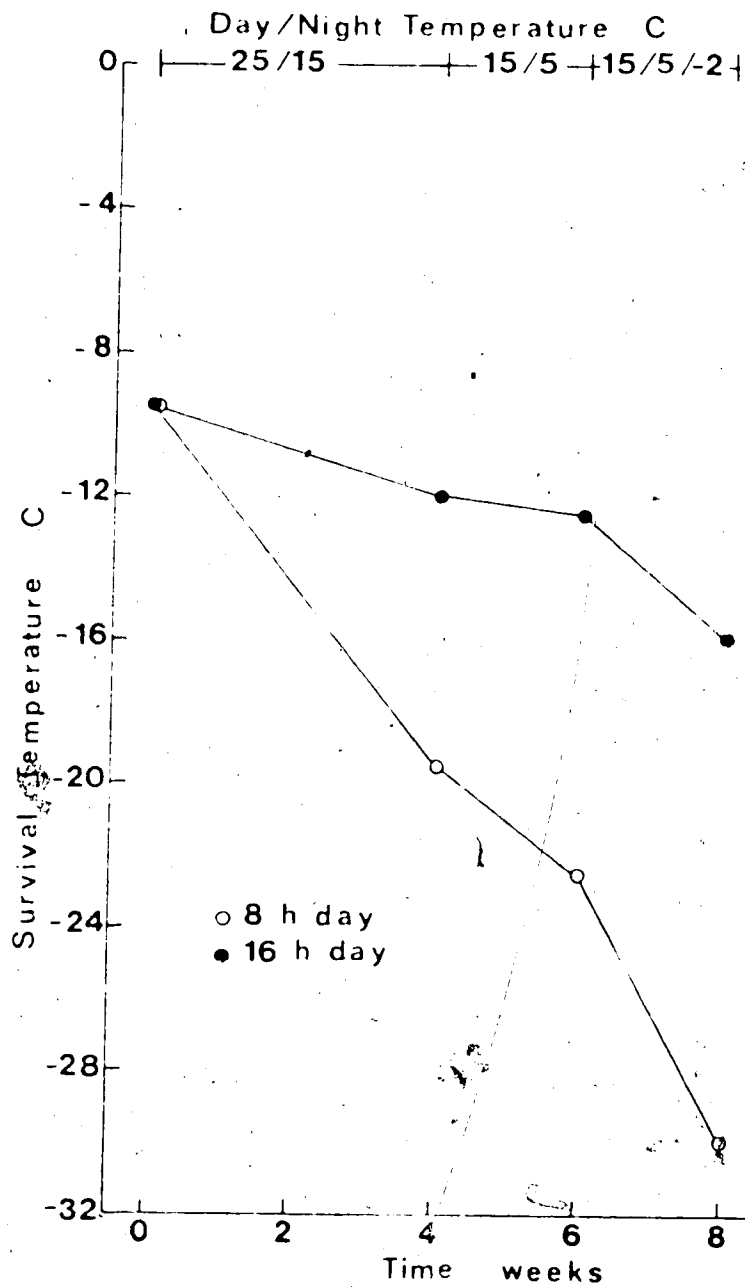
Fig. 8. The influence of photoperiod on the survival temperature of <u>Ledum groenlandicum</u>. Eight plants were subjected to each photoperiod regime. Three leafy shoots from each plant were used to determine the survival temperature.

value of -15.6 bars to -19.6 bars after 4 weeks at the 25/15 C temperature (see Fig. 9). After 2 weeks at the 15/5 C regime the total water potential of LD plants was -19.2 bars and a final value of -21.4 bars was measured after the 15/5/-2 C regime. The combined osmotic and matric potential of LD plants was initially -28.7 bars and was -25.9 bars after the first 4 weeks at the 25/15 C regime. The combined potentials decreased to -33.3 bars after 2 weeks at 15/5 C and increased to -24.9 bars after 2 weeks at 15/5/-2 C. Turgor pressure fluctuated during the study period; initially being 13.1 bars, decreasing to 6.4 bars after the first 4 weeks at 25/15 C, increasing to 13.9 bars after 2 weeks at 15/5 C, and finally decreasing to 3.5 bars after the 2 week 15/5/-2 C temperature regime.

The total leaf water potential of the SD plants decreased over the course of the study (see Fig. 9). From an initial reading of -15.5 bars, total water potential decreased to -16.1 bars after 4 weeks at 25/15 C, to -19.4 bars after 2 weeks at 15/5 C and then to -33.8 bars after the final 15/5/-2 C temperature regime. The combined osmotic and matric potential of SD plants followed a similar pattern to the total leaf water potential. At the outset of the study, the combined potential was -28.8 bars, increasing to -22.6 bars after 4 weeks, decreasing to -31.8 bars after the 2 week 15/5 C regime and decreasing to -44.4 bars after the final 2 week 15/5/-2 C temperature regime. As was found with the LD plants, turgor presuure fluctuated; from an initial
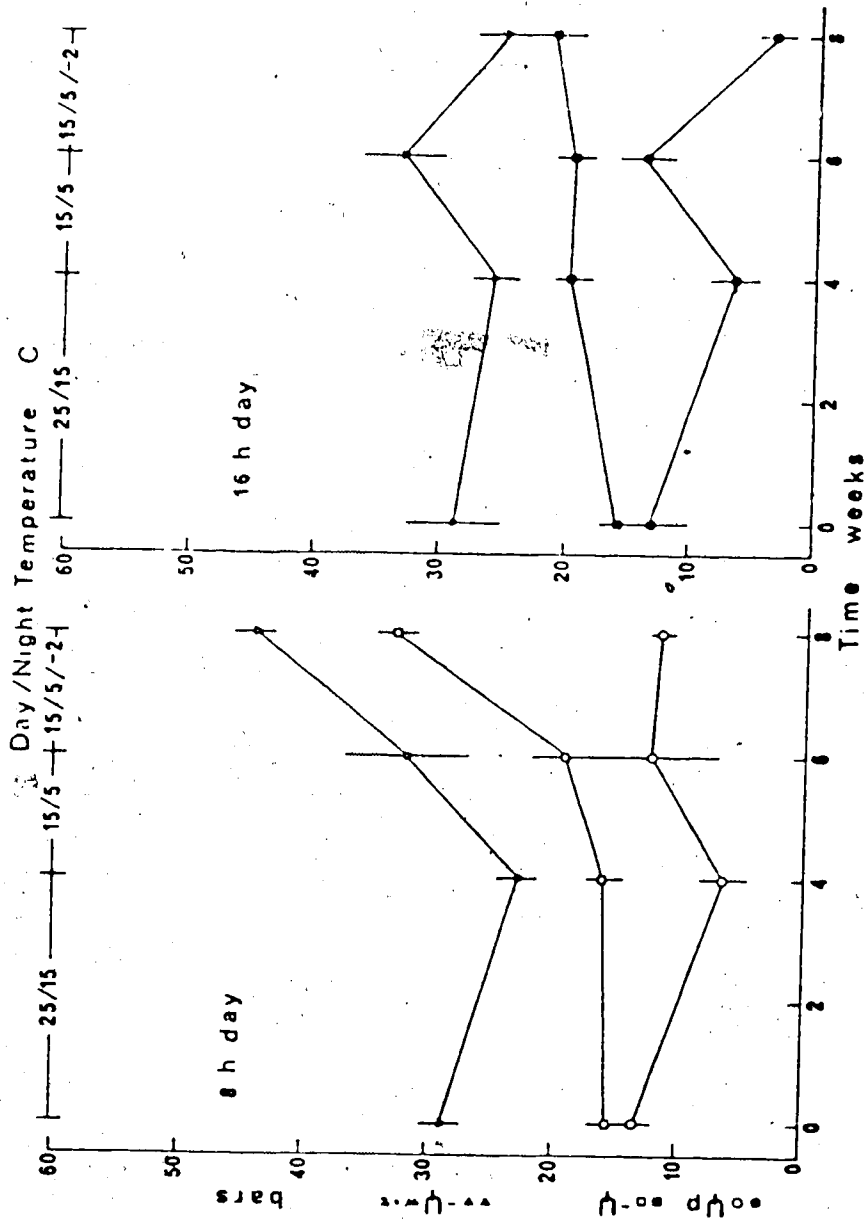
Fig. 9. The influence of photoperiod on the total leaf water potential (□), the combined osmotic and matric potential (▼), and the turgor pressure (○) of Ledum groenlandicum. Each symbol represents the mean of six plants. Vertical bars indicate the standard error of the mean.

13.4 bars, to 6.5 bars after 4 weeks, to 12.4 bars after 6 weeks, and to 11.6 bars by the eighth week of the study.

There was a significant difference between the total water potential and the component potentials of the LD and SD plants measured after the final 2 week period at the 15/5/-2 C temperature regime. Statistical significance was determined at the 0.01 level using an unpaired Student's t-test (eg. Zalik 1976).

The relative water content (RWC) of LD plants (see Fig. 10) did not change significantly over the course of the study. During the 8 week study period the initial RWC was .815 and the final value was .825. The RWC of SD plants (Fig. 10) did not vary significantly from the RWC of the LD plants until the eighth week of the study, when the SD plants RWC had decreased to .739. The RWC of the LD and the SD plants at 8 weeks were significantly different at the 0.01 level, determined by the unpaired Student's t-test (eg. Zalik 1976).

The Occurrence of Cavitation in Frozen Stems

The investigation into the occurrence of cavitation in the frozen stems of Ledum groenlandicum was prompted by the water relations results obtained during the field studies. Fig. 11 gives a comparison of the xylem tension (Scholander Bomb) values and total leaf water potential (psychometric) values gathered throughout the field study. During the
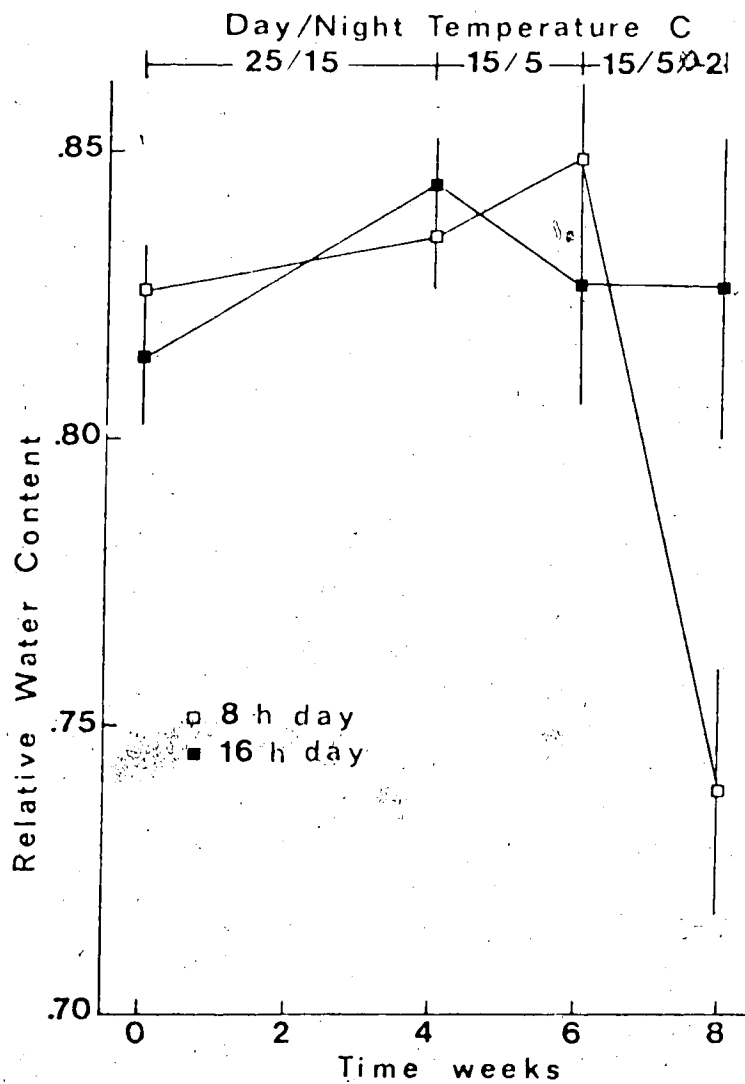
Fig. 10. The influence of photoperiod on the relative water content of Ledum groenlandicum. Each symbol represents the mean of six plants. Vertical bars represent the standard error of the mean.
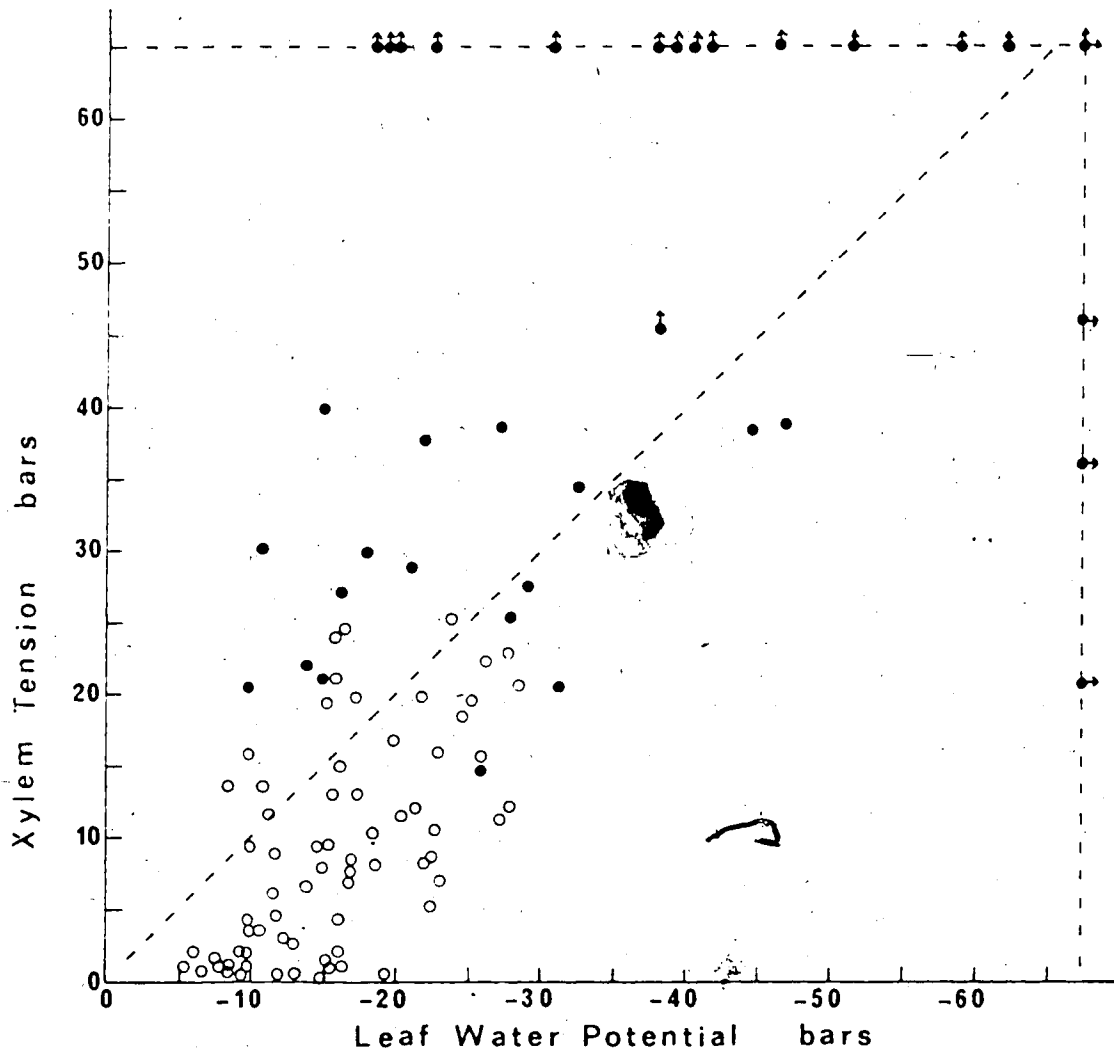
Fig. 11. The relationship between xylem tension (Scholander Bomb) and total leaf water potential (psychometric). Readings were taken at the Devonian Botanic Gardens. Closed symbols represent readings taken from November to March of each year. Open symbols are readings taken from April to October. Arrows indicate offscale readings.

winter months, xylem tensions were generally higher
(positive) than the total leaf water potential (negative)
values. An interpretation of these results was that during
the winter the frozen xylem sap had expanded and cavitated.
Because of the cavitation, the Scholander Bomb would have
required greater pressure to return the xylem sap to the cut
shoot surface, and therefore, xylem tensions would have been
erroneously high.


Water Uptake


The water uptake rates of cut shoots of Ledum
groenlandicum and Pinus banksiana were measured before and
after sections of the stems were frozen.

The cumulative water uptake of the four shoots of Ledum
groenlandicum are graphically portrayed in Fig. 12. Prior to
the freezing of the stems of Ledum groenlandicum, the rate
of water uptake of the cut shoots was 127.2 ± 13.2 (SE) ml
$g^{-1}$ fresh wt $h^{-1}$ (n=4). After the stem had been frozen and
thawed, the water uptake rate was reduced to 57.6 ± 7.8 ml
$g^{-1}$ fresh wt $h^{-1}$. The recovery rate of water uptake,
measured 24 h after freezing, was 35.5 ± 8.3 ml $g^{-1}$ fresh wt
$h^{-1}$. The water uptake rates of the individual shoots of
Ledum groenlandicum and Pinus banksiana are given in Table
8.

The cumulative water uptake of the four Pinus banksiana
shoots are portrayed in Fig. 13. The cut shoots of Pinus
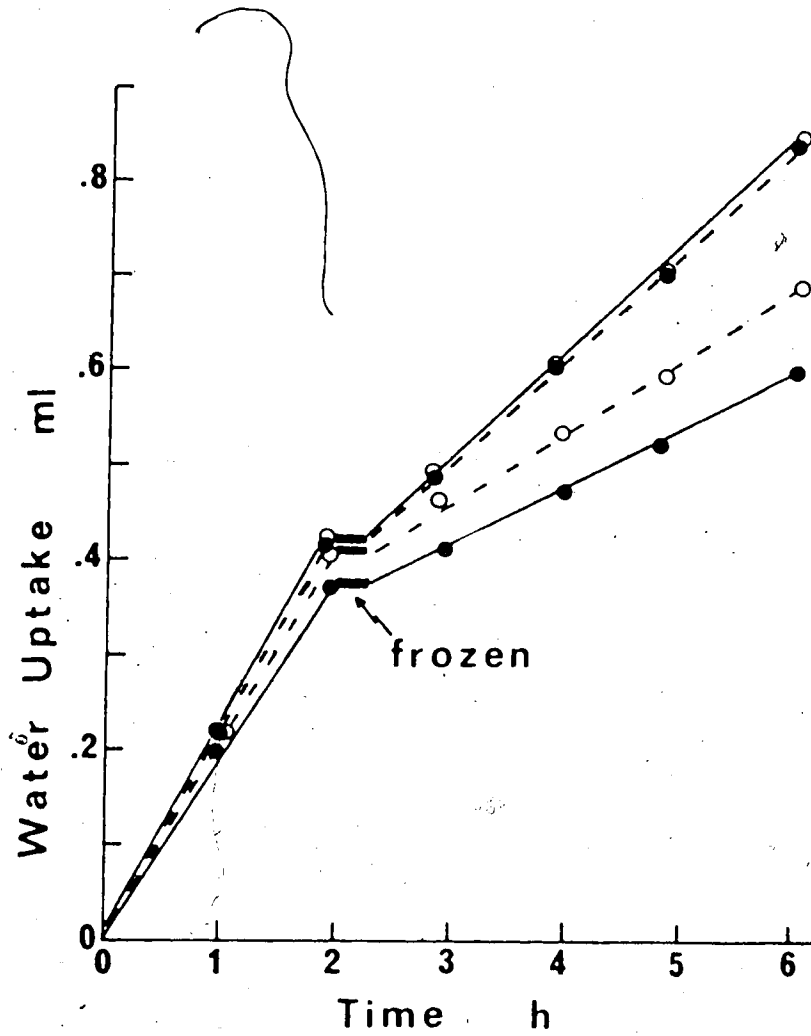
Fig. 12. The ᴉulative water uptake of <u>Ledum groenlandicum</u> shoots as a function of time. A 2.5 cm section of the stem was frozen with liquid $N_2$ for 15 min at 2 h.
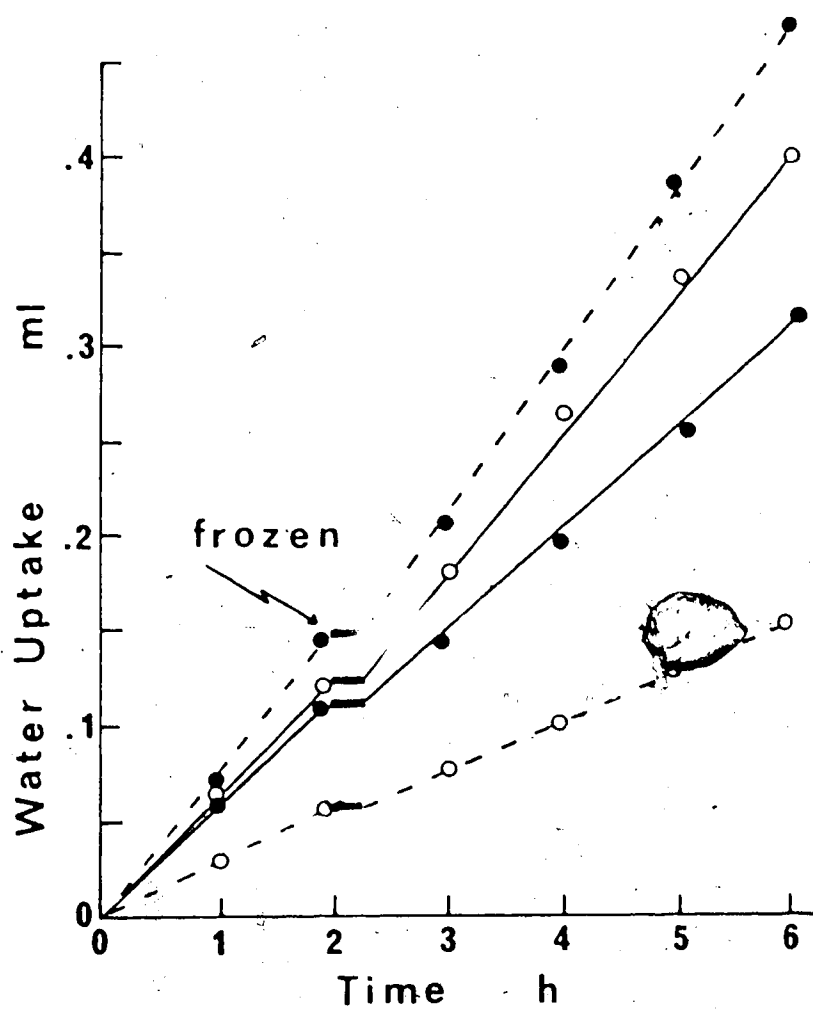
Fig. 13. The cumulative water uptake of Pinus banksiana shoots as a function of time. A 2.5 cm section of the stem was frozen with liquid $N_2$ for 15 min at 2 h.

Table 8.   The water uptake rates of individual shoots of
Ledum groenlandicum and Pinus banksiana before and after
freezing.

| Species | Shoot # | Water Uptake Rate ml g$^{-1}$ fresh wt h$^{-1}$ | | |
| --- | --- | --- | --- | --- |
| | | Before Freezing | After Freezing | After 24 h Recovery |
| L. groenlandicum | 1 | 115.2 | 62.8 | 47.4 |
| | 2 | 1 9.5 | 36.5 | 13.0 |
| | 3 | 117 6 | 57.2 | 48.8 |
| | 4 | 166.5 | 73.7 | 32.9 |
| P. banksiana | 1 | 18.8 | 18.9 | |
| | 2 | 18.8 | 21.2 | |
| | 3 | 21.1 | 24.7 | |
| | 4 | 12.0 | 10.9 | |

banksiana had much lower rates of water upt*e than the Ledum groenlandicum shoots. Prior to freezing of the stems of Pinus banksiana the water uptake rate was $17.7 \pm 2.0$ ml $g^{-1}$ fresh wt $h^{-1}$. Water uptake rates increased to $19.0 \pm 3.0$ ml $g^{-1}$ fresh wt $h^{-1}$ after the freezing and thawing of the shoots. It was not necessary to r~ ~d recovery rates at 24 h because the water uptake rate~ ~lowing freezing and thawing were not reduced.


Xylem Tension


The xylem tensions of frozen and unfrozen shoots of Ledum groenlandicum and Pinus banksiana were measured as another way to detect cavitation.

The xylem tension of Ledum groenlandicum shoots prior to the mid-day freezing period was $8.9 \pm 1.4$ (SE) bars (n=4). The stem was frozen for 10 min then thawed. After 7 h the post-freeze control shoots (shoots that had not been frozen) had a xylem tension of $4.5 \pm 0.5$ bars, indicating that the xylem water column was intact and the mid-day water deficit had been relieved. Shoots that had been frozen (post-experimental) had a xylem tension of $16.7 \pm 3.8$ bars at 7 h after the freezing period. The results indicated that the xylem water column of post-freeze experimental shoots had cavitated. Cavitation would disrupt the continuous·xylem water column, therefore, would impose a water deficit on the leaf tissue. The higher xylem tensions of post-freeze

experimental shoots may, therefore, be due to both the imposed water deficit and the development of air spaces in the xylem sap.

The xylem tension of the shoots of Pinυ banksiana was 11.8 ± 1.0 bars prior to the freezing riod. The post-freeze control shoots had a xylem tension of 11.0 ± 1.6 bars and the post-freeze experimental shoots had a xylem tension of 13.1 ± 1.2 bars. The results suggested that the post-freeze experimental shoots of Pinus banksiana had not cavitated.

There was a significant difference at the 0.05 level, using the unpaired Student's t-test (eg. Zalik 1976), between the post-freeze control and the post-freeze experimental xylem tensions of Ledum groenlandicum. The xylem tensions of the post-freeze control and the post-freeze experimental shoots of Pinus banksiana were not significantly different. The xylem tensions of the post-freeze control and experimental shoots of both species are presented as a percent of the xylem tension of the pre-freeze control shoots in    14.

The Effect of Tissue Hardiness on Höfler Diagrams

The relationship between the total leaf water potential and relative water content (RWC) for unhardened (-9 C) and hardened (<-40 C) leaf tissue is shown in Fig. 15. The shape of such curves has been shown to vary with the species and
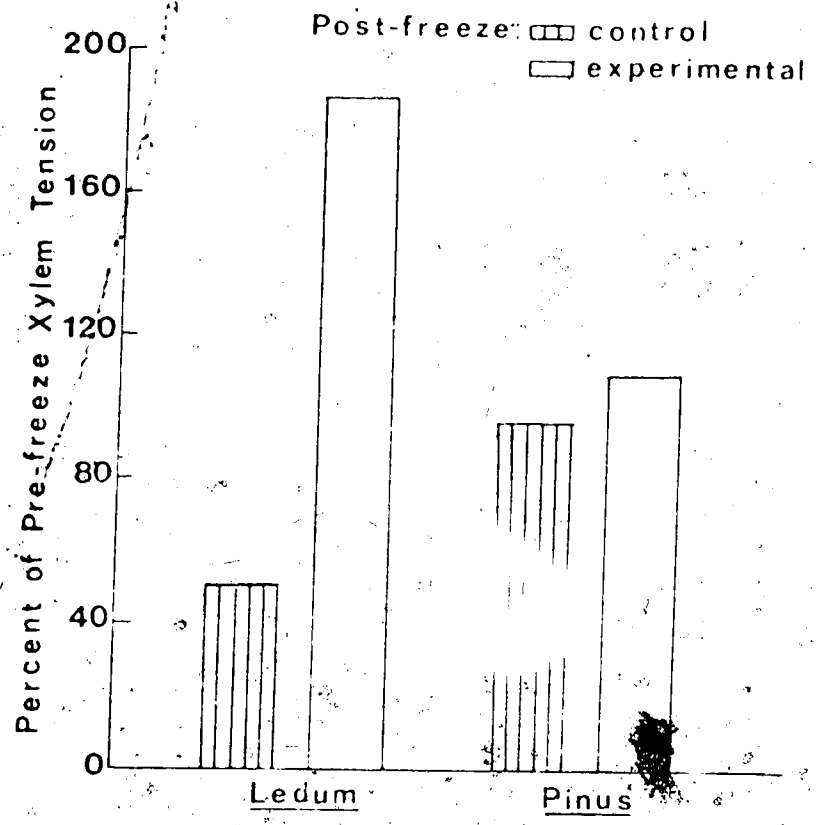
Fig. 14. The xylem tension of shoots of Ledum groenlandicum
and Pinus banksiana after recovery from freezing a 2.5 cm
stem section in situ for 10 min. Stems were frozen at noon
and post-freeze readings were taken 7 h after the freezing
event. Xylem tensions are expressed as a percent of the
xylem tensions of the pre-freeze control shoots. Values are
the mean of four plants.
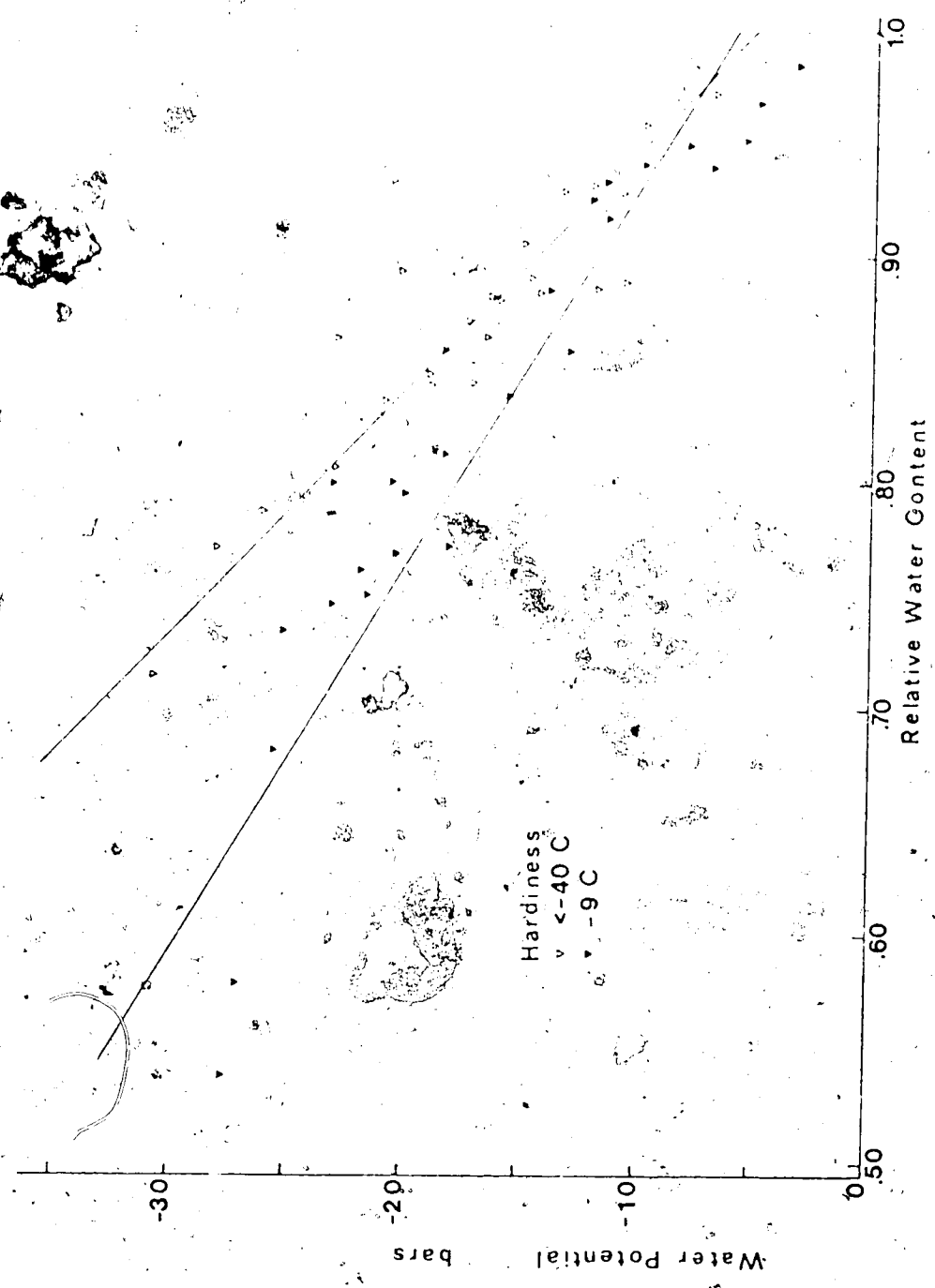
Fig. 15. The relationship between the relative water content and the total leaf water potential of unhardened (-9 C, ▼) and hardened (-40 C, ▽) Ledum groenlandicum.

growing conditions; xerophytes and plants growing under water stress tend to have steep curves. Fig. 15 shows that the hardened leaf tissue of Ledum groenlandicum had a steeper slope than the unhardened tissue, i.e. that the total leaf water potential was comparatively lower at a particular RWC.

To further characterize the internal water relations of Ledum groenlandicum tissue, the relationship between turgor pressure, combined osmotic and matric potential and RWC can be portrayed in a Höfler diagram as shown in Figs. 16 and 17.

## The Combined Osmotic and Matric Potentials

The combined osmotic and matric potential of Ledum groenlandicum leaf tissue at 1.0 RWC was -15.6 bars for unhardened tissue and -22.1 bars for hardened tissue (as calculated using Acock's (1975) model). The combined potentials of the unhardened tissue did not decrease as rapidly with a decrease the RWC as in the hardened tissue, indicating that with decreasing cell volume, there was a greater increase in the solute and colloidal concentration in hardened cells compared to unhardened cells. This analysis of the Höfler diagram was supported by the model proposed by Warren Wilson (1967a). Using the equations given in Table 7, p. 46 the osmotic potentials and matric potentials were calculated separately. Fig. 18 shows
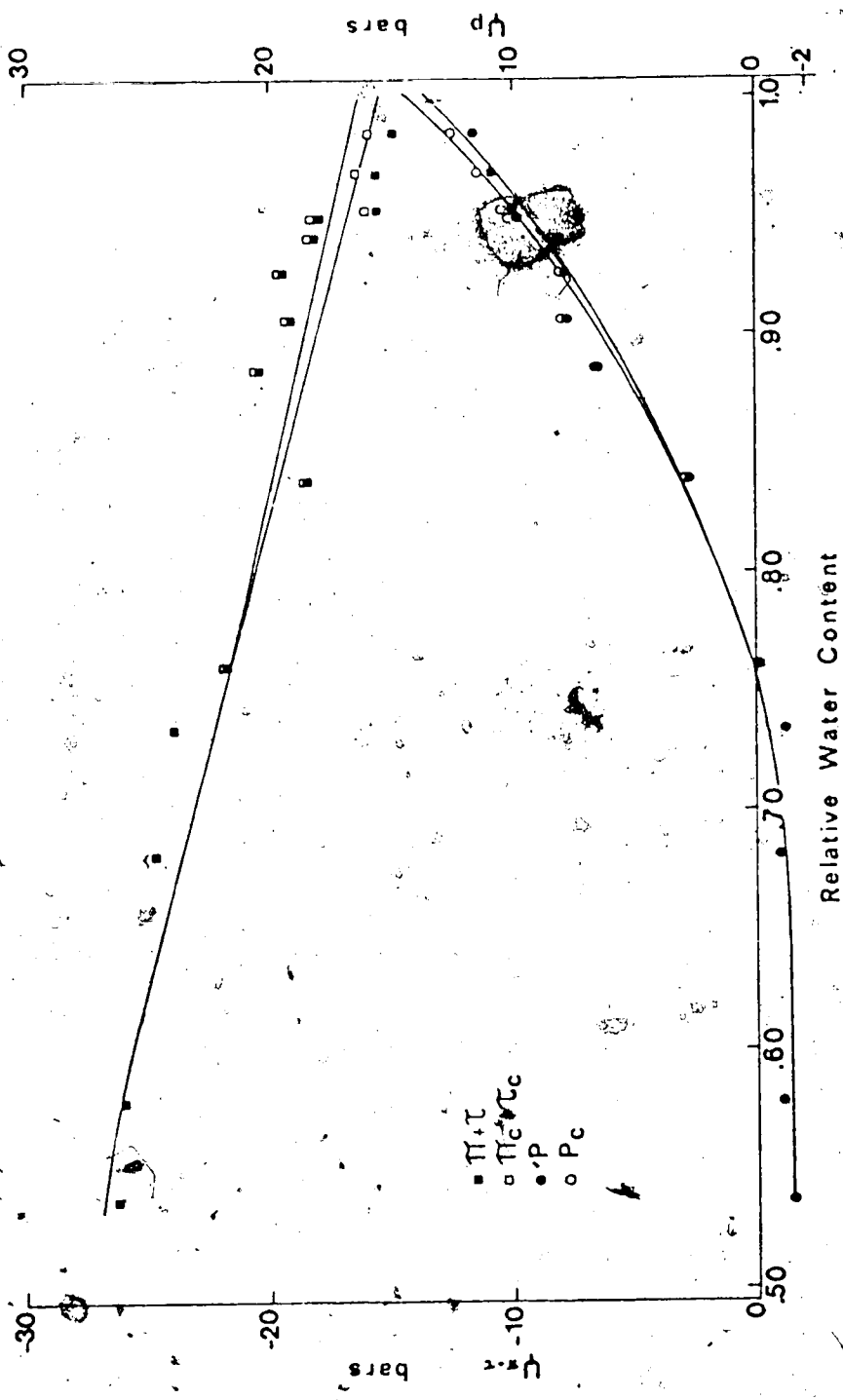
Fig. 16. The relationship between the relative water content and the water potential components of unhardened Ledum groenlandicum portrayed in a Höfler diagram. The closed symbols represent the measured bulk values of the combined osmotic and matric potential (■) and the turgor pressure (●). Open symbols are the intracellular component potential values that were calculated using the model by Acock (1975).
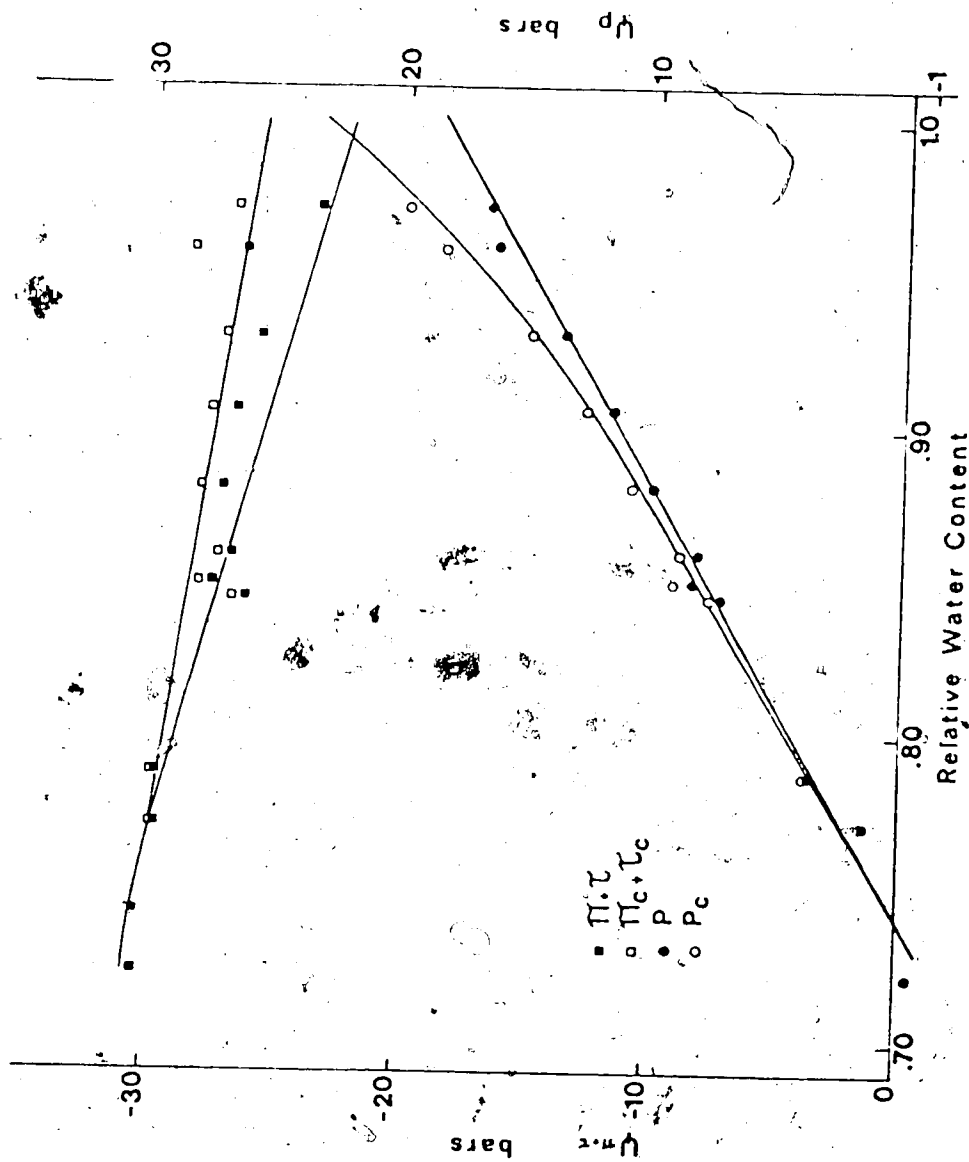
Fig. 17. The relationship between the relative water content and the water potential components of hardened *Ledum groenlandicum* portrayed in a Höfler diagram. The closed symbols represent the measured but values of the combined osmotic and matric potential (■) and the turgor pressure (●). Open symbols are the intracellular component potential values that were calculated using the model by Acock (1975).

that the hardened tissue had the lower osmotic and matric potentials at all RWC values, and they decreased more rapidly with a decrease in cell volume.

Calculated values of the bound water content (B), using the model of Warren Wilson (1967c), were .14 for unhardened tissue and .17 for hardened tissue. Based on Acock's (1975) model, where B was the y-intercept of the plot of R versus $1/\pi + \tau$, the bound water content for unhardened tissue was .183 and for hardened tissue was .253. The larger calculated bound water content value for hardened tissue supported the calculations of the greater matric potential of hardened tissue.

## Cell Wall Elasticity

The magnitude of the decrease in turgor pressure with a decrease in RWC is due to the relative elasticity of the cell walls. Walter (1931) conceptualized that plants with rigid cell walls had a rapid decline in turgor pressure with a small decrease in RWC, whereas plants cells having elastic cell walls maintained a relatively constant turgor pressure over a wide range in RWC. Plants having rigid cell walls were termed hydrostabile; plants with elastic cell walls were termed hydrolabile. Following Walter's (1931) terminology, unhardened plants of _Ledum groenlandicum_ had relatively elastic cell walls, therefore, were hydrolabile; hardened plants had rigid cell walls, therefore, were
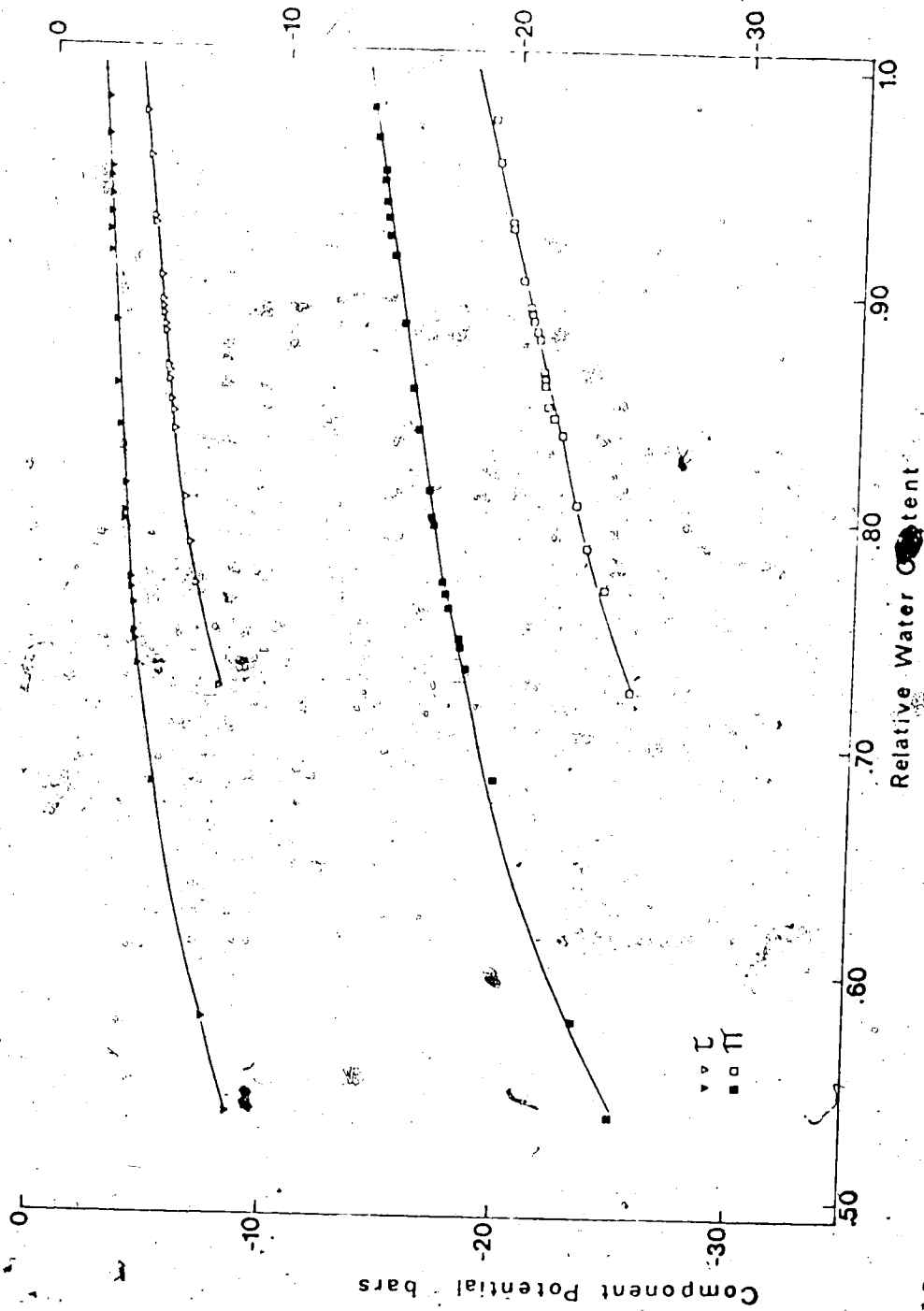
Fig. 18. The relationship between relative water content and the osmotic ($\pi$) and matric ($\tau$) potentials that were calculated using the model by Warren Wilson (1967a). Closed symbols represent unhardened (-9 C) _Ledum groenlandicum_ Open symbols represent hardened (-40 C) _Ledum groenlandicum_.

hydrostabile.

In rheological terms the modulus of elasticity of a substance is defined as (Scott Blair 1969):

$$\text{elastic modulus} = \frac{\text{stress}}{\text{strain}} \qquad (4)$$

Hydrostabile cells would have a higher elastic modulus because more force (or stress) would be required to produce a unit of deformation (or strain). Warren Wilson (1967c) derived a constant (e) (see Table 7, p. 46) that is similar to the elastic modulus, and is called the coefficient of enlargement. The calculated coefficient of enlargement of unhardened _Ledum groenlandicum_ cells was 36.6 bars, compared to the calculated value of 65.2 bars for hardened tissue. Table 9 lists the coefficients of enlargement, the bound water content and the combined osmotic and matric potentials at full turgor, as calculated by Warren Wilson (1967b), for a number of plant species.

Intracellular and Extracellular Water

Acock (1975) partitioned the commonly measured water potential components into intracellular water and extracellular water. A subscript 'c' in Figs. 16 and 17 denotes the intracellular component potentials calculated using the equations from Acock's (1975) model given in Table 7, p. 46. The model assumed that there was no turgor pressure component in the extracellular water potential. If

Table 9.    Estimates of    $\pi+\tau^*$, B, and e* for various species
as calculated by Warren Wilson (1967b), and for unhardened·
and hardened <u>Ledum groenlandicum</u> calculated
in this study.

| Species | $\pi+\tau^*$ | B | e |
|---|---|---|---|
| | bars | | bars |
| <u>Helianthus annus</u> | -3.1 | 0.27 | 7.3 |
| <u>Helianthus annus</u> | -10.0 | 0.26 | 27.7 |
| <u>Brassica napus</u> | -3.5 | 0.43 | 9.5 |
| <u>Zea mays</u> | -4.9 | 0.22 | 15.8 |
| <u>Gossypium hirsutum</u> | -11.5 | 0.00 | 28.6 |
| <u>Lycopesicon esculentum</u> | -11.0 | 0.11 | 21.5 |
| <u>Gossypium barbadense</u> | -16.9 | 0.22 | 29.6 |
| <u>Ligustrum lucidum</u> | -21.3 | 0.02 | 26.6 |
| <u>Pennisetum typhoides</u> | -15.9 | 0.00 | 33.4 |
| <u>Acacia aneura</u> | -22.1 | 0.00 | 84.3 |
| <u>Ledum groenlandicum</u> | | | |
| (unhardened) | -15.6 | 0.14 | 36.6 |
| (hardened) | -22.1 | 0.17 | 65.2 |

*  $\pi+\tau^*$=combined osmotic and matric potential at full turgor,
B= bound water content, $\epsilon$=coefficient of enlargement.

a calculated intracellular turgor pressure was greater than the measured bulk turgor pressure, Acock (1975) suggested that the extracellular water component had diluted the intracellular water during the freeze and thaw method commonly used to determine the component potentials (as on p. 29). If dilution of the intracellular solutes decreased the measured osmotic and matric potentials, then the calculated turgor pressure would also be decreased.

The difference between the calculated intracellular turgor pressure and the measured bulk turgor pressure for the unhardened Ledum groenlandicum tissue was small compared to the difference found in the hardened tissue. Based on Acock's (1975) model, these results indicated that the hardened Ledum groenlandicum tissue had a greater percent of its water partitioned as extracellular water.

## The Effect of Tissue Hardiness on Transpiration

The influence of light and dark regimes on the transpiration rate of hardened and unhardened shoots of Ledum groenlandicum was studied. The hardiness of the tissue had a noticeable effect on transpiration rates (see Fig. 19).

Unhardened tissue had a transpiration rate of about 242 ± 26 (SE) mg g$^{-1}$ dry wt h$^{-1}$ (n=8) during the initial light period. During the dark period, the transpiration rate decreased over a 2 h period to 37 ± 8 mg g$^{-1}$ dry wt h$^{-1}$.
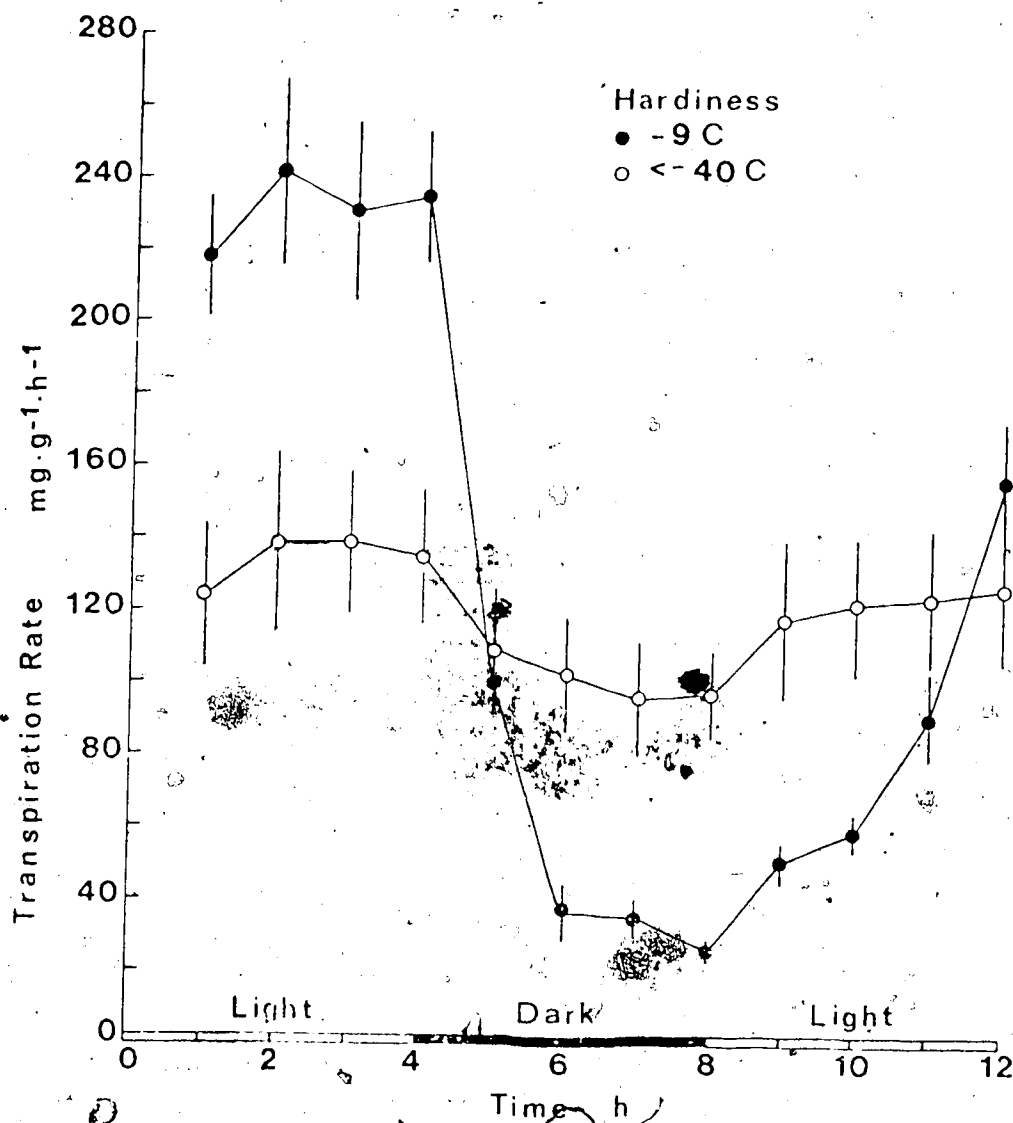
Fig. 19. The transpiration rates, in response to dark and light periods, of unhardened and hardened Ledum groenlandicum. Air temperature was 20 C. Symbols represent the mean, of eight shoots. Vertical bars indicate the standard error of the mean.

When the light period was resumed there was a sluggish increase toward the initial light period transpiration rate.

The transpiration rate of hardened <u>Ledum groenlandicum</u> during the initial light period was about $139 \pm 20$ mg g$^{-1}$ dry wt h$^{-1}$. There was a very slow and small decrease in the transpiration rate to $103 \pm 16$ mg g$^{-1}$ dry wt h$^{-1}$ during the dark period. The final light period caused a slow increase to the intial transpiration rate. The small decrease in transpiration rate during the dark period could have been due to the decrease in leaf temperature. Leaf temperature decreased 1 to 3 C when the growth chamber lights were switched off. The dark period transpiration rate of the hardened shoots was about three times greater than that of the unhardened shoots.

## The Effect of Tissue Hardiness on Net Photosynthesis

The Effect of Temperature on Net Photosynthesis

The effect of leaf temperature on the net photosynthetic rates of hardened and unhardened <u>Ledum groenlandicum</u> is given in Fig. 20.

Unhardened plants had grown under a 25/15 C temperature regime and reached a maximum net photosynthetic rate of 4.06 .40 (SE) mg $CO_2$ dm$^{-2}$ h$^{-1}$ (n=4) at 25 C. Net photosynthesis was negative below 1.0 C.

Hardened plants, that had grown under a 15/5/-2 C
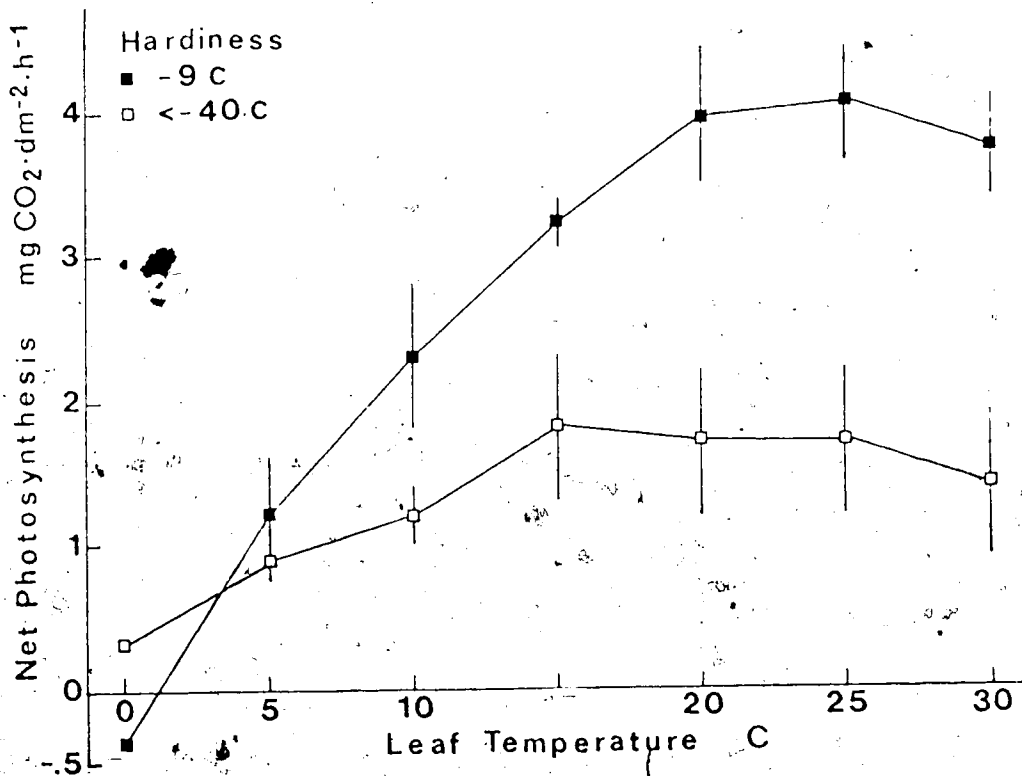
Fig. 20. The net photosynthesis - temperature response
curves for unhardened and hardened Ledum groenlandicum.
The light intensity was 800 µE m-2 s-1. Each symbol
represents the mean of four plants. Vertical bars indicate
the standard error of the mean.

temperature regime, reached a maximum net photosynthetic rate of 1.77 ± .50 mg $CO_2$ $dm^{-2}$ $h^{-1}$ at 15 C. Net photosynthesis was positive at 0 C, and by extrapolation, the minimum temperature for positve net photosynthesis was -3 C.


The Effect of Temperature on Dark Respiration


The dark respiration rates of hardened and unhardened Ledum groenlandicum plants increased with temperature as shown in Fig. 21. The $Q_{10}$ values calculated for the interval between 15 C and 25 C were 2.2 and 2.8 for hardened and unhardened plants respectively. Dark respiration rates were greater at all temperatures in the hardened plants.

The Arrhenius plots for the dark respiration rates are given in Fig. 22. Lyons (1972,1973) found that the slope of the Arrhenius plots of the respiration rates of chilling sensitve plants increased at temperatures below 10 to 12 C. The Arrhenius plots of both the hardened and unhardened plants of Ledum groenlandicum had constant slopes over a range from 0 to 30 C, indicating that neither group of plants were chilling sensitive.


The Effect of Light Intensity on Net Photosynthesis


The net photosynthesis-light intensity response curves for unhardened and hardened Ledum groenlandicum are given in
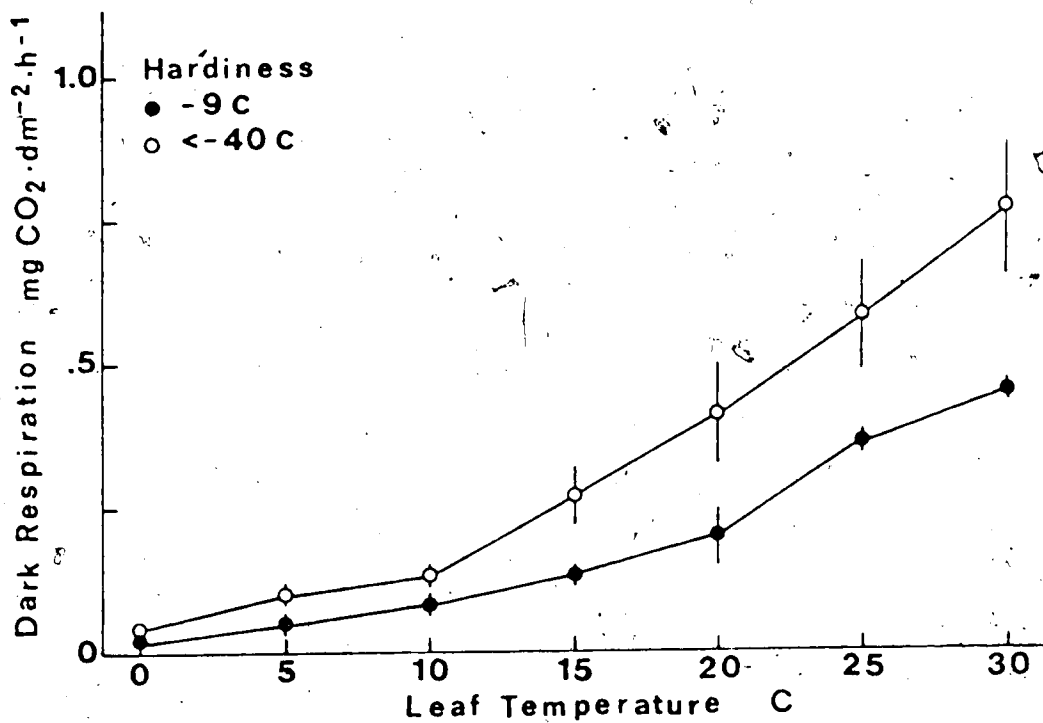
Fig. 21. The dark respiration - temperature response curves for unhardened and hardened <u>Ledum groenlandicum</u>. Symbols represent the mean of three plants. Vertical bars indicate the standard error of the mean.
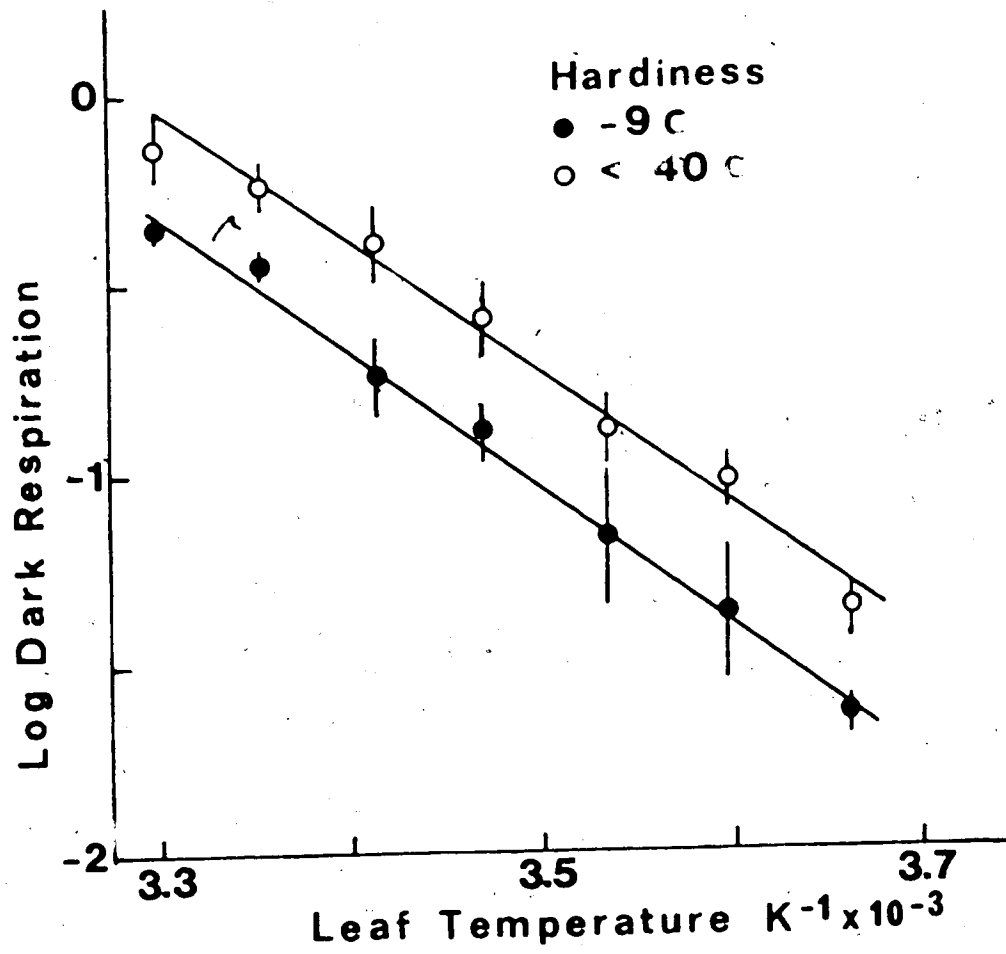
Fig. 22. The Arrhenius plots of the dark respiration rates versus leaf temperature for unhardened and hardened Ledum groenlandicum. Each symbol represents the mean of three plants. Vertical bars indicate the standard error of the mean.

Fig. 23. The light intensity response curves were determined at the temperature for optimum net photosynthesis of the two hardiness levels (see p. 82). The light compensation point of both unhardened and hardened plants was 20 $\mu E$ $m^{-2}$ $s^{-1}$. Because the light compensation point increases exponentially with temperature, hardened plants would have had a higher compensation point than the unhardened plants if both had been measured at 25 C.

Both hardened and unhardened plants showed a similar response to increasing light intensity. The net photosynthetic rate of unhardened plants increased to a maximum of 3.02 ± .15 (SE) mg $CO_2$ $dm^{-2}$ $h^{-1}$ (n=3) at a light intensity of 1050 $\mu E$ $m^{-2}$ $s^{-1}$. Over the range of light intensities used, the net photosynthetic rate for hardened tissue reached a maximum rate of 2.25 ± .47 mg $CO_2$ $dm^{-2}$ $h^{-1}$ at 1450 $\mu E$ $m^{-2}$ $s^{-1}$. The light intensity response curve of both the unhardened and hardened plants approached the light saturation level at about 800 $\mu E$ $m^{-2}$ $s^{-1}$.
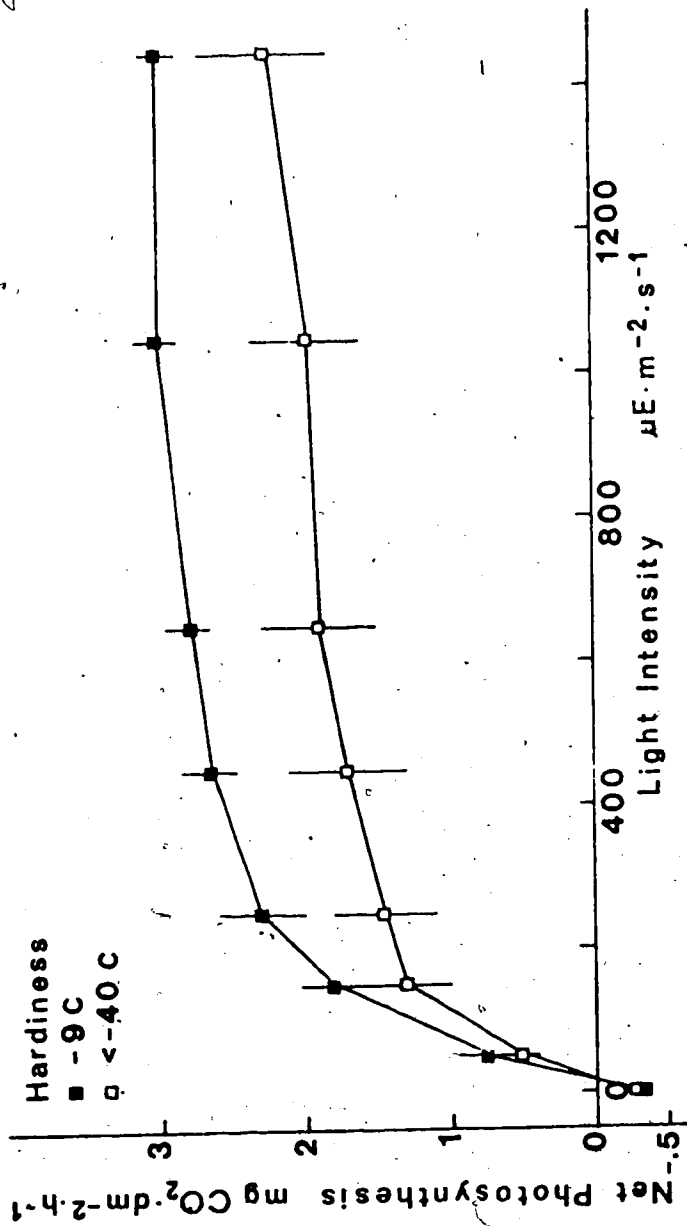
Fig. 23. The net photosynthesis - light intensity response curves for unhardened and hardened Ledum groenlandicum. Unhardened plants were held at 25 C, hardened plants at 15 C. Each symbol represents the mean of three plants. Vertical bars indicate the standard error of the mean.

# DISCUSSION

Ledum groenlandicum developed a cold tolerance to temperatures below the minimum temperature of its habitat. The plants growing at the University of Alberta Devonian Botanic Gardens survived a minimum air temperature of -45.5 C in January 1976 and -33.9 C in December 1976. The retention of a survival temperature of -9 C throughout the summer of 1976 safeguarded the plants against unseasonal frosts. Mild frosts did occur in late May and early June 1976.

Based on the results of this study, the cold acclimation of Ledum groenlandicum, from the relatively tender summer condition to the hardy winter condition, is summarized in Table 10. The findings of this study support Weiser's (1970) hypothesis for the cold acclimation of hardy woody plants (see Table 1, p. 10). The first stage of acclimation is induced by short days accompanied by warm temperatures. During this stage, growth ceases and metabolic changes occur that promote the second stage of acclimation. The first stage of acclimation does not cause a large increase in the plant's cold hardiness. The first stage of acclimation in the field study plants occurred between the sampling dates of September 12, 1976 and October 9, 1976, when daylength became shorter than 13 h, maximum

Table 10. Summary of time and events of cold acclimation in Ledum groenlandicum based on the results of this study.

| Spring | Summer | Early Autumn | Late Autumn | Winter |
|---|---|---|---|---|
| daylength >13 h warm days >20 C | long days warm temperature | daylength <13 h warm days >20 C cool nights 5-10 C | short days frosts | prolonged subfreezing temperatures |
| hardiness inhibitor (cytokinin) ? | hardiness inhibitor (cytokinin) ? | hardiness promoter (abscisic acid) ? | ↑ membrane permability ↑ stomatal resistance ↑ root resistance | cavitation of xylem sap |
| $\psi \sim -14$ bars $\psi p \sim 5$ bars | $\psi \sim -10$ bars $\psi p \sim 4.5$ m | $\psi \sim -12$ bars $\psi p \sim 4.5$ bars | $\psi \sim -25$ bars $\psi p \sim 2.5$ bars | $\psi < -65$ bars $\psi p \sim .5$ bars |
| rapid growth | slow growth→growth stops (phytochrome) ? | | dormant | quiescent |

photosynthates accumulate ————→ increased solutes and colloids

————→ protoplasmic augmentation

increased respiratory capacity

elastic cell walls ————————————————————→ rigid cell walls

increased apoplastic water ————————————→

| tender (-12 C) | tender (-9 C) | first stage of acclimation (-27 C) | second stage of acclimation (<-40 C) | hardy (<-40 C) |

temperatures were above 20 C (see Fig. 3, p. 50) and the survival temperature decreased from -10.7 C to -23 C (see Fig. 4, p. 52). The first stage of acclimation was also observable in the controlled environment photoperiod study (see Fig. 8, p. 59). During the first 6 weeks of the study, maximum temperatures were 15 C or above and SD plants hardened from -9 C to -22.5 C. Long day plants hardened to -12.5 C.

There is debate as to whether light has a photosynthetic role or a photoperiodic role in the first stage of acclimation (eg. Alden and Hermann 1971). In September 1976, air temperatures were warm and field study Ledum groenlandicum plants were relatively tender, therefore, net photosynthesis would have approached optimum rates if one considers the net photosynthesis-temperature response curve shown in Fig. 20, p. 83. Terminal buds had formed by August 19, 1976 on Ledum groenlandicum. This suggests that the photosynthates produced during the late summer and early fall were not utilized in growth, but were accumulated and used in the active metabolic pathways initiated during the first stage of acclimation.

The requirement for a short photoperiod during the first stage of acclimation was substantiated by the results obtained in the controlled environment study (Fig. 8, p. 59). However, whether the short day photoperiod initiated a phytochrome response is not certain. Williams, Pellet, and Klien (1972) and MacKenzie et al. (1974a) found that

end-of-the-day far-red light exposure after long days promoted growth cessation and a small increase in cold hardiness in Cornus stolonifera. Dark periods interrupted by red flashes supressed the cold acclimation of Cornus stolonifera and Weigelia florida, but had no effect on Pyracantha coccinea (Williams et al. 1972). The spectral ratio of far-red:red light for twilight measurements made by van Zinderen Bakker (1974), 20 km ESE of Edmonton at intervals throughout 1973-74 are given in Table 11. As is expected in an area at high latitudes having long periods of summer twilight, the far-red:red ratio was highest in June and July. A critical interpretation of the results of McKenzie et al. (1974a) and the results of the present study suggests that the role of far-red light, via the phytochrome system, is to initiate growth cessation and dormancy, however, it may be independent of the role of the short day photoperiod. Once growth cessation occurs, the short day photoperiod initiates the first stage of acclimation, perhaps by activating another translocatable factor. Abscisic acid has been implicated as the translocatable factor in the hardening of Acer negundo (Irving 1969). Kacperska-Palacz, Debaska, and Jakubowska (1975) found that red light increased the cold hardiness and increased the rate of removal of water from rape seed tissue. Perhaps the red light stimulated the formation of ABA in the leaves, which in turn increased the tissue membrane permeability. Attempts to isolate ABA from the leaf tissue of Ledum

Table 11.  The far-red (750 nm):red (650 nm) spectral ratios
measured at twilight 20 km ESE of Edmonton, Alberta
throughout 1973-74 (from van Zinderen Bakker 1974).

| Date | Time  h | Ratio | Comments |
|------|---------|-------|----------|
| May 6 and 7 | 20:00 | .95 | clear |
| May 29 and 30 | 20:00 | 1.00 | clear |
| June 27 and 28 | 21:00 | 1.06 | cloudy |
| July 21 and 22 | 20:00 | 1.06 | overcast |
| August 18 and 19 | 20:00 | .76 | cloudy |
| September 6 and 7 | 18:00 | .93 | clear |
| October 2 and 3 | 18:00 | .75 | clear |
| October 17 and 18 | 18:00 | 1.00 | overcast |
| April 18 and 19 | 17:00 | .94 | overcast |

groenlandicum were unsuccessful (see Appendix E, p. 145),
however, its possible role in the cold acclimation processes
cannot be ignored.

The second stage of acclimation (Weiser 1970) is
induced by subfreezing temperatures. It involves physical,
rather than metabolic, processes and results in a rapid
increase in cold hardiness. The field study plants were
exposed to frost by October 9, 1976. After this time,
subfreezing temperatures became more persistent and severe
and Ledum groenlandicum rapidly hardened to below -40 C.
Plants grown under SD and subjected to -2 C frosts each
night for 2 weeks under a controlled environment (see Fig.
8, p. 59) also hardened rapidly to below -30 C. Long day
plants hardened to only -16 C. These results support the
hypothesis (Weiser 1970) that a SD photoperiod is required
during the first stage of acclimation to trigger the
metabolic changes that ready the plant for the second stage
of acclimation.

Dehardening in the spring is reported to be induced by
warm weather (Alden and Hermann 1971). Timmins and Worrall
(1974) found evidence of a translocatable dehardening
promoter in 'climatically split' Douglas fir. Menhennet and
Wareing (1977) suggested that a plant's dehardening response
to temperature and daylength in the spring might be mediated
by endogenous cytokinin levels. In both the spring of 1976
and 1977, Ledum groenlandicum dehardened following a period
of maximum temperatures above 20 C; daylengths greater than

13 h coincided at both times. Spring growth flushes and dehardening occurred simultaneously in 1976, but not in 1977, suggesting that these were independent events.

Prior to both the spring growth flush and dehardening, leaf water potential increased, therefore, both processes required an increase in the tissue hydration. Dennis, Carpenter, and MacLean (1975) found that there was a rapid spring dehardening of sour cherry flower buds when they reached the 'water bud stage', i.e. they began to swell due to tissue hydration. The turgor pressure of Ledum groenlandicum (Fig. 7, p. 57) reached a maximum value prior to the flush of growth and remained high throughout the growth period. As turgor pressure decreased, the growth rate also declined. Hsiao (1973) stressed the importance of turgor pressure as the driving force for extension growth. The optimum turgor pressure during the rapid growth of Ledum groenlandicum was lower than the maximal value, agreeing with the concept that full turgor will not yield maximum growth (Mayo personal communication). A water potential gradient must be maintained so that there is a water flux into the cell acting as the driving force for growth.

Ledum groenlandicum plants were not water stressed throughout the summer or early fall of 1976. These results agreed with those of Small (1972). He measured xylem tensions of 17 ± 2 bars and a stomatal resistance of 1.2 ± 0.4 s cm$^{-1}$ in Ledum groenlandicum growing in a raised Sphagnum peat bog on a hot dry summer day. Hartgerink and

Mayo (unpublished data) found maximum midsummer xylem tensions of Ledum groenlandicum were between 14 and 15 bars.

Summer and early fall drought have been shown to decrease the cold hardiness of Castanea sativa, Diosyros kaki, Juglans regia, Paulownia tomentosa, Acer velutinum (Borzakivs'ka and Majko 1965), and Scotch pine (Eiche 1966). It is probable that a drought during the first stage of acclimation would inhibit the necessary photosynthate accumulation and metabolic processes that are required for the second stage of acclimation.

Gates (1914) and Small (1972) claimed that the xeromorphy found in several ericaceous shrubs is an adaptation to survive winter desiccation. During late September and October there was little precipitation at the Devonian Botanic Gardens and the available soil moisture would have been expected to decrease. It has been found that plants have an increased tolerance to frozen soils if the soil moisture is low (Calder, MacLeod, and Jackson 1965, Kilpatrick et al. 1966, Timmins and Tanaka 1976). Ledum groenlandicum field study plants also showed decreased hydration levels and increased cold hardiness in October. Drought stress may promote an increase in cold hardiness in Ledum groenlandicum, but the drought stress may be induced by physiological mechanisms that respond to environmental factors such as photoperiod or temperature, rather than soil moisture. As shown in Figs. 9 and 10 (p. 61 and 63), a significant dehydration of leaf tissue occurred in well

watered plants grown under SD. hardening conditions. Cornus stolonifera plants grown under SD, warm temperature, well watered conditions also had a significant reduction in the stem tissue water content and an increased stem cold hardiness (Chen et al. 1975). It was found in both Ledum groenlandicum and Cornus stolonifera that LD conditions had no significant effect on the tissue hydration level or cold hardiness.

The decreased hydration level in Cornus stolonifera has been attributed to an increased root resistance and a decreased stomatal resistance during acclimation (Chen et. al. 1975, McKenzie et al. 1974b). The water absorption capacity of sunflower and tomato roots was found to decrease at low temperatures (Kramer 1940). The decreased water absorption rates was a combination of the decreased root membrane permeability and the increased viscosity of water at low temperatures. Increased suberization of roots with age may also increase root resistance (Kramer and Bullock 1966). The transpiration rate of fully hardened tissue of Ledum groenlandicum in the light was much lower than unhardened tissue (Fig. 19, p. 81), indicating that the stomatal resistance of hardened Ledum groenlandicum had increased. Increased stomatal resistance was also found in hardened Picea excelsa, Pinus sylvestris (Christerrson 1972), Rhododendron catawbiense, Kalmia latifolia, and Pinus nigra (Parker 1963a). The stomatal resistance of Ledum groenlandicum was measured with a diffusive resistance

porometer, in the field on March 20, 1977. Five of the seven readings were offscale (i.e. were calibrated to be greater than 30 s cm$^{-1}$). At this time the survival temperature of Ledum groenlandicum was below -40 C. The porometer results, therefore, support the controlled environment study findings that hardened Ledum groenlandicum had a high stomatal resistance in the light. The increased transpiration rate of hardened Ledum groenlandicum in the dark (see Fig. 19, p. 81), however, may promote tissue dehydration.

A mechanism similar to that found for the chill-hardening of Phaseolus vulgaris (Wilson 1976) may be operative during the cold acclimation of Ledum groenlandicum. Wilson (1976) found that when Phaseolus plants grown at 25 C, 85% R.H. were transferred to a 5 C, 85% R.H. chamber, the stomata opened temporarily and the root permeability decreased. Recovery, and then a further increase in stomatal resistance occurred after 6 h in the low temperature chamber. An intial opening of the stomata and a decreased root permeability of Ledum groenlandicum plants exposed to freezing temperatures during cold acclimatiol may occur. This process would account for the tissue denycration found in field plant: in October (Fig. 5, p. 54) ·· · in the SD plants after the last 2 weeks of the controlled environment regime (p. 58 and Fig. 9, p. 61).

The role of SD in the induction of tissue dehydration is unclear. Short days may control the hormonal balance so that there is a compartmentalization of cytokinins and ABA.

If root cytokinin levels were high, to decrease root permeability; leaf mesophyll tissue ABA levels were high, to increase the membrane permeabililty; but, guard cell ABA levels were low to ensure open stomata, then tissue dehydration could feasibly occur. This process is speculative, because cytokinin activity in <u>Nicotina rustica</u> shoots was rapidly inactivated by water stress (Itai and Vaadia 1971). Short day photoperiods reduced the cytokinin level in the leaves and buds of <u>Xanthium strumarium</u> (Henson and Wareing 1977a). Henson and Wareing (1977b) suggested that the reduction of leaf cytokinin levels was due to the rapid transmission of a signal to the roots that either depressed the synthesis or the release of the hormone. Compartmentalization of ABA in the leaves of <u>Xanthium strumarium</u> has been suggested by Raschke, Pierce, and Popiela (1976) to explain anomalous water relations results.

The mild dehydration to about -30 to -40 bars during October in the <u>Ledum groenlandicum</u> field plants and to -30 bars in the controlled environment study plants may be due to the aforementioned process. However, the severe dehydration occurring during periods of prolonged subfreezing temperatures was likely due to xylem sap cavitation. On several occasions during the winter, xylem tension was much higher than the corresponding negative leaf water potential (see Fig. 11, p. 64). Measurement of a high xylem tension in comparison to the corresponding leaf water potential has been found for a number of specie (Klepper

and Ceccato 1969, Boyer 1967, Kaufman 1968a, Tobiesson, Rundel, and Stecker 1971, Barker 1973, Hellkvist, Richards, and Jarvis 1975). Cavitation has been implicated as the cause for the discrepancy between the xylem tension and water potential in a number of cases. Laboratory studies indicated that the Ledum groenlandicum xylem sap cavitated following a freezing stress (Figs. 12 and 14, p. 66 and 71). Cavitation has been shown to occur in the xylem elements of grapevine (Scholander, Love, and Kanwisher 1955), tropical lianas (Scholander, Ruud, and Leivstad 1957), Ricinus (Milburn 1973), Plantago (Milburn and McLaughlin 1974), and Malus sylvestris (West and Gaff 1976) due to moderate water stress. Lybeck (1959) found that freezing the sap of maple caused the release of dissolved gases in the conducting elements.

Pinus banksiana xylem sap did not show signs of cavitation after freezing (Figs. 13 and 14, p. 67 and 71). Hammel (1967) hypothesized that gymnosperms do not cavitate readily because the bordered pits of the tracheids maintain a postive pressure during the melting of the frozen sap, and thus, impede the formation of air bubbles. The open ended vessels that predominate in the vasculature of an angiosperm would be unable to confine a pressure gradient, and therefore, upon melting the xylem sap would be under tension and the outgassed bubbles would expand. Metcalfe and Chalk (1957) described the vessels of Ledum groenlandicum as 'not particularly long'. The vessel diameter is extremely small

(<25 u) and there are 200 or more vessels per square mm. The vessel perforation plates are scalariform. The cavitation in Ledum groenlandicum may be impeded because of the wood anatomy, however, the perforation plates would not be capable of maintaining a pressure gradient between the vessels.

If the Ledum groenlandicum plants were covered by a thick layer of snow, then they would be subject to relatively stable temperature conditions in a high relative humidity environment and cavitation of the xylem sap would not impose any severe desiccation problems. The winters of 1975-76 and 1976-77 were relatively dry, with a light snow cover and many above freezing days. The branches of Ledum groenlandicum that rose above the snow cover were exposed to a great variation in temperature. Xylem sap cavitation undoubtedly occurred at subfreezing temperatures. The transpiration that would have occurred during warm daytime periods, either via the stomatal or cuticular pathway, in combination with the low water absorption capacity of the plant, would have resulted in the tissue dehydration found from November to March of 1975-76 and 1976-77 (see Figs. 5 and 6, p. 54 and 55).

The xylem sap cavitation must have been relieved because the water balance improved each spring prior to the flush of growth. Hygen (1965) found that there was slow water transport in a small portion of the conducting elements of a frozen Norway spruce stem. He concluded that a

supercooled fraction of the xylem water (maintained a constant rate of water absorption and conduction at subfreezing temperatures. Mayo (unpublished data) found well-watered Ledum groenlandicum attained a root pressure of 1.9 cm Hg, a pressure that would be capable of raising water to a height of 26.8 cm. The water balance of Ledum groenlandicum improved after snow melt when soil was close to saturation, therefore, cavitation may have been repaired by root pressure alone, or in combination with the growth of new vascular tissue.

The decreased osmotic and matric potentials found in hardened Ledum groenlandicum in the field and in the controlled environment studies was as expected. A decrease in the osmotic potential of overwintering plants has been found in several studies (see Table 3, p. 23). The decrease in the osmotic and matric potential of Ledum groenlandicum was due to a decrease in the tissue relative water content as shown in the controlled environment study (Fig. 10, p. 63). The Höfler diagram for hardened tissue (Fig. 17, p. 75) and the analysis of the data using Warren Wilson's (1967a) model (Fig. 18, p. 77) indicated that the decreased osmotic and matric potential was also due to an increase in the solute and colloidal concentration, regardless of the water content. The increased solute and colloidal fraction of the cell might occur during the active metabolic first stage of acclimation. During the augmentation of the protoplasm in hardening tissue (as described by Siminovitch et al. 1968)

there is an increase in the concentration of soluble sugars and proteins. The augmentation of the protoplasm of Ledum groenlandicum would, therefore, decrease the combined osmotic and matric potential.

The calculated turgor pressure of Ledum groenlandicum tissue during the winter was negative on several occasions. Based on the analysis of water potential isotherms, Tyree (1976) claimed that negative turgor pressures could not exist. His theory may explain the occurrence of negative turgor pressures in Ledum groenlandicum. Both Acock (1975) and Tyree (1976) stated that at equilibrium, the apoplastic (extracellular) and symplastic (intracellular) leaf water potentials are the same. According to Acock (1975):

$$\Psi = \pi c + \tau c + P c = \pi w + \tau w \qquad (5)$$

where
$$\Psi = \text{total leaf water potential,}$$
$$\pi c + \tau c = \text{intracellular osmotic and matric potential,}$$
$$P c = \text{intracellular turgor pressure, and}$$
$$\pi w + \tau w = \text{extracellular osmotic and matric potential.}$$

In the development of Acock's (1975) model, he assumed that there was no turgor pressure component in the extracellular water, and negative potentials developing in the cell wall were due only to matric forces and solutes. Tyree (1976) stated that negative pressures develop in the

apoplastic water due to the surface tensions of the water in the evaporating surfaces of the cell wall. These tensions propagate through the cell wall water that is held outside the Donnan free space. The osmotic and matric potentials of the apoplastic water would be negligible except very locally inside the Donnan free space. Therefore, Tyree (1976) claimed that most of the apoplastic water is relatively pure, but it is held under tension.

Both Acock (1975) and Tyree (1976) had no objections to measuring the total leaf water potential with a thermocouple psychrometer. However, both agreed that upon freezing and thawing of the leaf tissue, there is an exchange between the apoplastic and the symplastic water. When turgor pressure is greater than zero, both visualized a movement of apoplastic water into the symplastic water to give a diluted, therefore, an underestimated value of $\pi c + \tau c$ and thus, an underestimated calculated turgor pressure.

According to the equation (5) p. 103 from Acock's (1975) model, if $Pc$ is negative, then upon freezing and thawing, the intracellular water will be more dilute than the extracellular water and there will be a reversal of the dilution effect. This does not seem probable, especially when in the present study, negative turgor pressures were found cold hardened _Ledum_ _groenlandicum_ exposed to su... temperatures. The occurrence of a dilute intra... water fraction in hardened tissue would promote ... cellular freezing.

Tyree (1976) stated that measured negative turgor pressures are due to /the diffusion of symplastic solutes into the relatively pure apoplastic water following the freeze and thaw process. Negative turgor pressures of -2 bars were extrapolated from the Höfler diagram of unhardened Ledum groenlandicum (Fig. 16, p. 74). From the analysis of the cell walls of the unhardened plants (p. 76) the cell wall volume was low, the cell walls were relatively elastic, and the apoplastic water content was minimal. With these characteristics, only small apparent negative turgor pressures would be measured because the apoplastic water would have only a small dilution effect. The Höfler diagram for hardened Ledum groenlandicum (Fig. 17, p. 75) suggested that large negative turgor pressures could hav developed. The analysis of the cell walls of hardened plants (p. 76) revealed that the cell wall volume was large, cell walls were rigid, and the proportion of apoplastic water was high. When the turgor pressure approached zero, there would have been a large dilution effect of the symplastic water, resulting in an apparent large negative turgor pressure. During the field study, negative turgor pressures were measured only in hardened plants subjected to subfreezing temperatures.

The possibility that negative turgor pressures existed in Ledum groenlandicum cannot be ignored. Levitt and Zaken (1975) suggested that the development of a negative turgor pressure would decrease the cell volume, but increase the

cell surface area. An increased cell surface area would be advantageous for the fast removal of intracellular water to extracellular ice nucleation sites. Negative turgor pressures arising in unhardened, actively metabolizing cells would propagate the tension and deformation of the protoplasmic proteins' tertiary structures and ultimately lead to death (Tyree 1976, Hsaio 1973). The protoplasm of hardened tissue becomes elastic and resistant to dehydration (Weiser 1970). This may be due to changes in protein configurations (Levitt 1972) or protoplasmic augmentation (Siminovitch et al. 1968) and would increase the tissue's resistance to the stresses incurred by protoplasmic tens..ns upon dehydration and perhaps positive pressures upon rehydration (McKenzie et al. 197ac).

The highest leaf water potential value measured in Ledum groenlandicum during the course of this study was -3.4 bars. The extrapolated values of the leaf water potential at 1.0 RWC from the Hofler diagrams (Figs. 16 and 17, p. 74 and 75) were -1.5 bars and -3.2 bars for hardened and unhardened tissue respectively. According to the equation for leaf water potential (eq. 5, p. 103) given by Acock (1975), leaf water potential can never be zero because the apoplastic water always has a negative potential. Tyree (1976) assumed there was also a negative pressure component in the cell walls, therefore, his model also supports the concept that zero water potentials cannot occur.

The increase in the coefficient of enlargement of the

cell walls of <u>Ledum</u> <u>groenlandicum</u>, from 36.6 bars for unhardened tissue to 65.2 bars for hardend tissue, suggests that cold acclimation increases the rigidity of cell walls. Parker (1963b) proposed that cold resistant plants had inflexible cell walls. Jarvis and Jarvis (1963) found that drought resistant spruce and pine had more rigid cell walls than drought intolerant birch and aspen. The increased cell wall rigidity in hardened <u>Ledum</u> <u>groenlandicum</u> may not be the direct result of cold or drought acclimation. Kassam and Elston (1976) found that the coefficient of enlargement (e) increased with age in the leaves of <u>Vicia</u> <u>faba</u>. Knipling (1967) and Kaufman (1968b) attributed the decrease in cell wall elasticity of older leaves to the increase in dry matter. Although a decrease in the elasticity may be due to leaf aging, it could have secondary advantages for cold hardened tissue. Gaff aand Carr (1961) found an increase in the dry wt:fresh wt ratio in plants of <u>Eucalyptus</u> <u>globulus</u> after they were exposed to a drought stress. They suggested that a thickened cell wall is a consequence of 'hardening off', and thus, increases the buffering capacity of the wall against transient water conditions.

The larger cell wall volume of hardened <u>Ledum</u> <u>groenlandicum</u> tissue would give a greater apoplastic water volume in relation to the symplastic water volume. This would be advantageous in the avoidance of intracellular freezing in the hardened tissue. A larger cell wall volume would give a larger specific surface of the cell protoplast

(or less volume strain per unit surface at any one degree of cell contraction), and thus, decrease the likelihood of mechanical injury during the freeze-induced dehydration (Levitt 1972). The intercellular space of tissue that has rigid cells would increase with a decrease in turgor pressure (Levitt and Zaken 1975), therefore, would increase the area for extracellular ice formation. Mazur (1969) hypothesized that the cell walls in hardened tissue must be relatively elastic so that the plasmolysed protoplasm is not under an extreme tension. However, a rigid cell wall, as found in hardened Ledum groenlandicum, would snap back with less force upon rehydration, and thus, lessen the chances of tearing the protoplasmic surfaces during deplasmolysis.

Older leaves can develop cuticular cracks (Nobel 1974) that could lower the cuticular resistance and increase the transpiration rate. The transpiration rate of hardened Ledum groenlandicum (Fig. 19, p. 81) was constant over the light and dark periods. This could be due to a hormonal regulation of the stomata (possibly ABA) causing a sluggish response to changing light regimes (Christersson 1972). It is possible that the stomatal resistance could be as high as that for the unhardened tissue in the dark, and the discrepancy in the water flux was due to water moving out of the leaf via the cuticular pathway. However, Sifton (1963) found that the cuticle of Ledum groenlandicum increased in its thickness and resistance to disintegration with age because of the progressive polymerization of the cutin.

The net photosynthetic rate of unhardened Ledum groenlandicum measured at 25 C was 3.98 ± 0.41 mg $CO_2$ g⁻¹ dry wt h⁻¹ or 4.06 ± 0.40 mg $CO_2$ dm⁻² h⁻¹. Mooney (1972) reported the normal maximum photosynthetic rates of the Ericaceae to be between 4 and 12 mg $CO_2$ dm⁻² h⁻¹. Smith and Hadley (1974) measured rates of 3.83 ± 0.20 mg $CO_2$ g⁻¹ dry wt h⁻¹ for Ledum groenlandicum acclimatized to a 30 C light, 25 C dark thermal regime. The net photosynthetic rate of hardened tissue was reduced by, and showed a thermal acclimation to the 15 C day temperature. The maximum net photosynthetic rate was 1.77 ± 0.50 mg $CO_2$ dm⁻² h⁻¹ at 15 C. The thermal acclimation of photosynthesis has been shown in Ledum groenlandicum (Smith and Hadley 1974), Quercus rubra (Chabot and Lewis 1976), Pinus taeda (Strain, Higginbotham, and Mulroy 1976), Eucalyptus pauciflora (Slatyer and Murrow 1977) and several plant species from diverse origins (Mooney and West 1964), but has not been shown in some species such as pitch pine (Ledig, Clark, and Drew 1977).

Hadley and Bliss (1964) found a reduction in the net photosynthesis of old shoots of Ledum groenlandicum at the end of the growing season and attributed it to the withering of the leaves. The reduction of net photosynthesis in Ledum groenlandicum in this study may be due to the increased dark respiration rates of hardened tissue (see Fig. 21, p. 85). Smith and Hadley (1974) found that the dark respiration rates of Ledum groenlandicum increased following cold acclimation. Cold growing temperatures were found to

increase the dark respiration rates of subterranean clover (Fukai and Silsbury 1977), Hordeum vulgare (Svec and Hodges 1973), Rhododendron catawbiense, Kalmia latifolia, and Pinus nigra (Parker 1963a). An increase in the respiratory capacity of the cold hardened Ledum groenlandicum plants explains the increase found in their light compensation points at 25 C compared to unhardened plants. The increased respiratory cap ity of plants during cold hardening is attributed to the increase in the mitochondrial fraction accompanying the augmentation of the protoplasm (Siminovitch et al. 1968).

The decrease in the net photosynthetic rates of hardened Ledum groenlandicum in this study is probably not due solely to the increased respiratory capacity. Hadley and Bliss (1964) found that in the winter, Ledum groenlandicum growing on Mt Washington had negligible net photosynthesis. Respiration rates at 3 C were reduced to 0.02 mg $CO_2$ $g^{-1}$ dry wt $h^{-1}$. Smith and Hadley (1974) suggested that the photosynthetic and respiratory mechanisms in Ledum groenlandicum acclimatiz d independently. Gifford (unpublished data) found that there were no changes in the levels of chlorlophyll a and b and α-carotene in overwintering leaves of the ericaceous Arctostaphylos uva-ursi. No build up in the metabolites of photosynthesis were found, suggesting that the key enzymes of photosynthesis were not inhibited. Chloroplast were found to be intact throughout the winter in leaves of Buxus and

Ilex (Parker 1963b). Gifford (unpublished data) suggested that photosynthesis in the winter may be reduced by the inhibition of transcription and translation. Ledum groenlandicum is similar in its overwintering habit to Arctostaphylos uva-ursi and probably undergoes similar changes in its photosynthetic mechanisms. Net photosynthesis in Ledum groenlandicum, therefore, may be partly reduced because of a slow down in the rates of synthesis and destruction of components of the photosynthetic system.

The decreased transpiration rates found in hardened Ledum groenlandicum shoots (see Fig. 19, P. 81) suggest that an increased stomatal resistance to $CO_2$ diffusion may contribute to the reduction of net photosynthesis in hardened plants. The cell wall resistance to $CO_2$ would also have increased in hardened tissue. Carbon dioxide encounters a resistance as it diffuses through the water filled interstices of the cell wall (Nobel 1974) and the resistance encountered is proportional to the average thickness of the walls. The average thickness of the cell walls of hardened Ledum groenlandicum was greater than that for unhardened tissue. The plants were well owatered throughout the net photosynthesis studies, and Acock (1975) stated that large amounts of water in the cell walls of thick walled species would increase the diffusion path for $CO_2$, and thus, increase the mesophyll resistance.

Reduced rates of net photosynthesis and respiration would also be expected in hardened, dehydrated plants at

subfreezing field temperatures. The $CO_2$ mesophyll resistance has been shown to increase at temperature extremes (Rawson, Begg, and Woodward 1977) and at low water potentials (Bunce 1977, O'Toole et al. 1976). The inhibition of the photosynthetic and respiratory enzymatic reactions by low temperature would also be important. The temperature for positive net photosynthesis in controlled environment studies of hardened Ledum groenlandicum was -3 C. Positive net photosynthesis at subfreezing temperatures has been found in a number of cold hardy plants (see Table 2, p. 15).

If the photosynthetic mechanism was intact and potentially functional in overwintering Ledum groenlandicum, then with the onset of warm temperatures and a decrease in the stomatal resistance in the spring, the plants would be capable of immediately fixing carbon. The high respiratory capacity of the tissue could function in mobilizing the reserve materials that were synthesized during the augmentation of the protoplasm in the fall and render them available for new spring growth.

# CONCLUSIONS

_Ledum groenlandicum_ is hardy to temperatures below the
minimum seasonal environmental temperatures (i.e. <-40 C).
Its cold acclimation in the fall is accomplished in at least
two stages. The first stage of acclimation requires a short
photoperiod and above freezing temperatures. The leaf water
potential remains high and the increase in hardiness is
small. The second stage of acclimation is triggered by
subfreezing temperatures. The leaf tissue becomes severely
dehydrated and rapidly increases in hardiness. Plants must
undergo the first stage of acclimation before they can
adequately harden during the second stage.

The xeromorphic characteristics of _Ledum groenlandicum_
are probably adaptations to the severe winter drought
conditions. _Ledum groenlandicum_ does not experience a water
stress at any time during the summer or early fall months.
The tissue dehydration during the cold acclimation is partly
due to the drier environmental conditions, but is also due
to changes in the plants' physiological mechanisms in
response to photoperiod and temperature. A short day
photoperiod may alter the plant's hormonal balance that
regulates root and stomatal resistances. Cavitation of the
xylem sap, following continual freezing and thawing periods,
combined with periods of high transpiration throughout the

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winter, imposes a severe dehydration stress on the plant tissue. Alleviation of the water stress occurs in the spring prior to dehardening and the spring flush of growth.

The survival temperature of _Ledum groenlandicum_, in a habitat having such wide seasonal variation in its water availability is due to _Ledum groenlandicum_'s ability t undergo physiological adaptations. Turgor pressure rises to optimal levels during rapid growth stages in the spring, and total leaf water potential · remains high throughout the summer and early fall. The cell walls of unhardened _Ledum groenlandicum_ are relatively elastic, therefore, throughout the summer and early fall _Ledum groenlandicum_ is hydrolabile and turgor pressure remains constant over a wide range of relative water contents. Hardened tissue has rigid cell walls, therefore, in the late fall and winter _Ledum groenlandicum_ becomes hydrostabile. Turgor pressure falls off rapidly with a decrease in the relative water content and the stomata would be expected to close. The proportion of apoplastic water increases in hardened tissue, therefore, there is an increase in the avoidance of intracellular freezing. Both the increased cell wall rigidity and the increased fraction of apoplastic water in hardened tissue make it more resistant to the mechanical stresses accompanying plasmolysis and deplasmolysis that occur during freezing and thawing.

The photosynthetic capacity of hardened _Ledum groenlandicum_ is decreased to at least 50% of that of

unhardened tissue. This could partly be due to the high dark respiration rates found in hardened tissue. The stomatal resistance and mesophyll resistance of hardened tissue also increased, therefore, the flux of $CO_2$ into the chloroplast would decrease. Low temperature inhibition of the photosynthetic reaction rates and the inhibition of the synthesis and destruction of the components of the photosynthesis system may also play a role. The increased capacity of dark respiration in hardened Ledum groenlandicum and the increased osmotic and matric potentials in hardened Ledum groenlandicum supports the concept that during hardening the tissue undergoes protoplasmic augmentation.

Several areas that were cursorily touched on in this investigation warrant further study. Among the areas in which questions have arisen are:

1) the role of phytochrome and endogenous hormones (particularly ABA) in the cold acclimation mechanism,

2) the seasonal variation in the root resistance, stomatal resistance, and photosynthesis,

3) the possible role of negative turgor pressure in overwintering tissue,

4) the changing properties of the cell/wall, and

5) the possible role of protoplasmic augmentation.

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# APPENDIX A

Graphs of a thermocouple psychrometer calibration curve and

a leaf disc saturation curve.

Diagrams of a leaf disc saturation apparatus and a
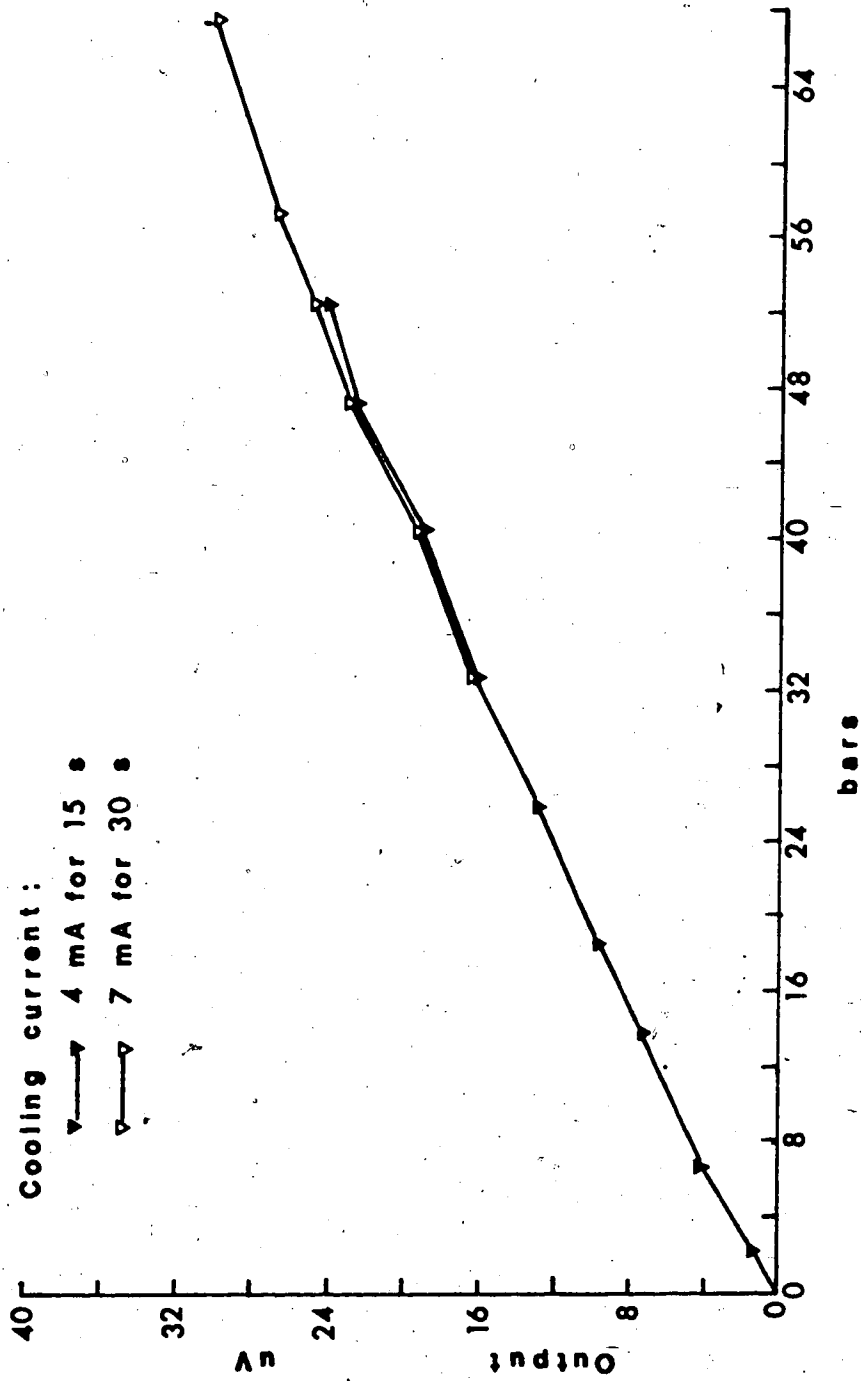
potometer.

Fig. 24. An example of a thermocouple psychrometer calibration curve used to convert the Fluke voltmeter output to water potential in bars.
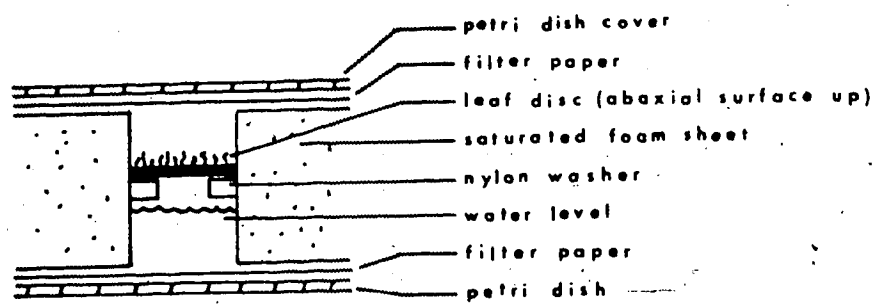
Fig. 25.  Diagram of the leaf disc saturation apparatus used in the relative water content determinations of <u>Ledum</u> <u>groenlandicum</u>. (2X actual size)
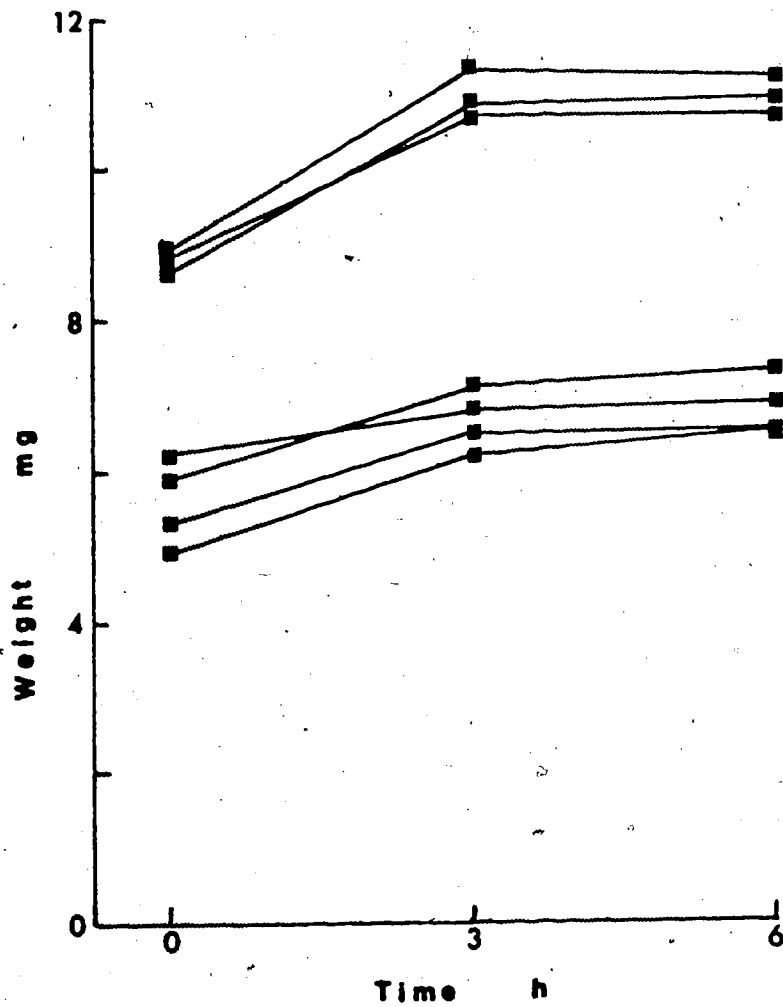
Fig. 26. Graph of the increase in leaf disc fresh weight
with time during the saturation period of relative water
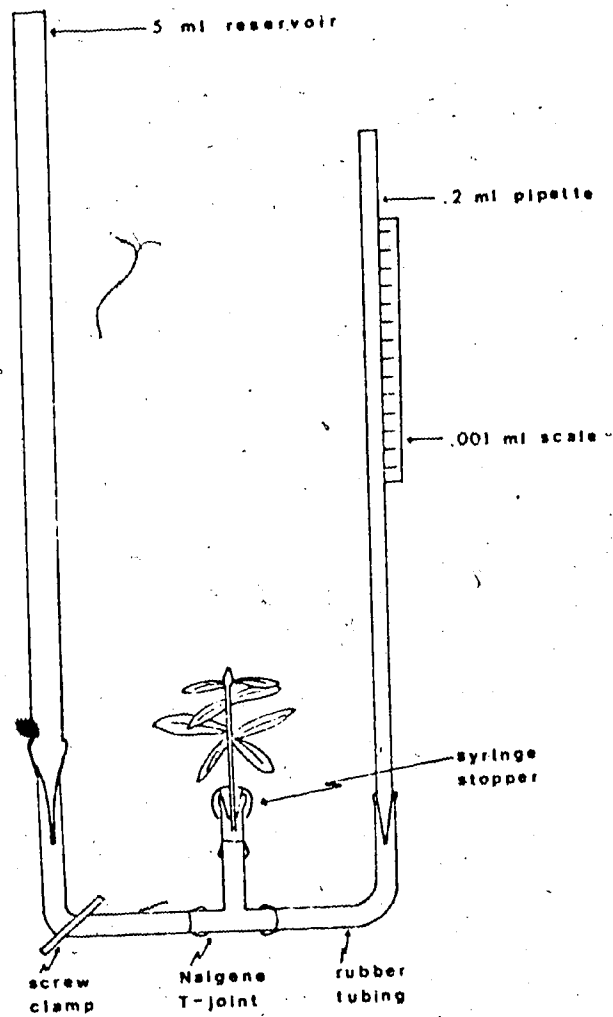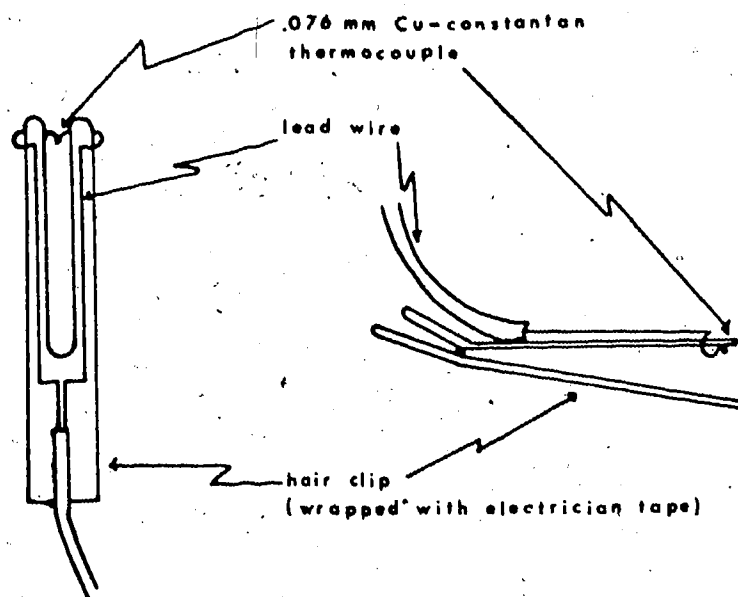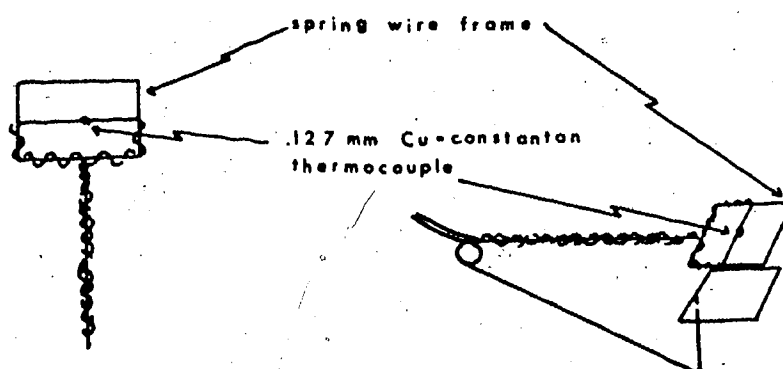content determinations.

Fig. 27.   Diagram of potometer used to measure the
transpiration rates of cut shoots of Ledum groenlandicum
and Pinus banksiana.

## APPENDIX B

Diagrams of leaf thermocouples, cuvette, and the IRGA gas
flow system.

a. .076 mm Cu-constantan thermocouple used in cold hardiness determinations.



b. .127 mm Cu-constantan thermocouple used in leaf temperature determinations.
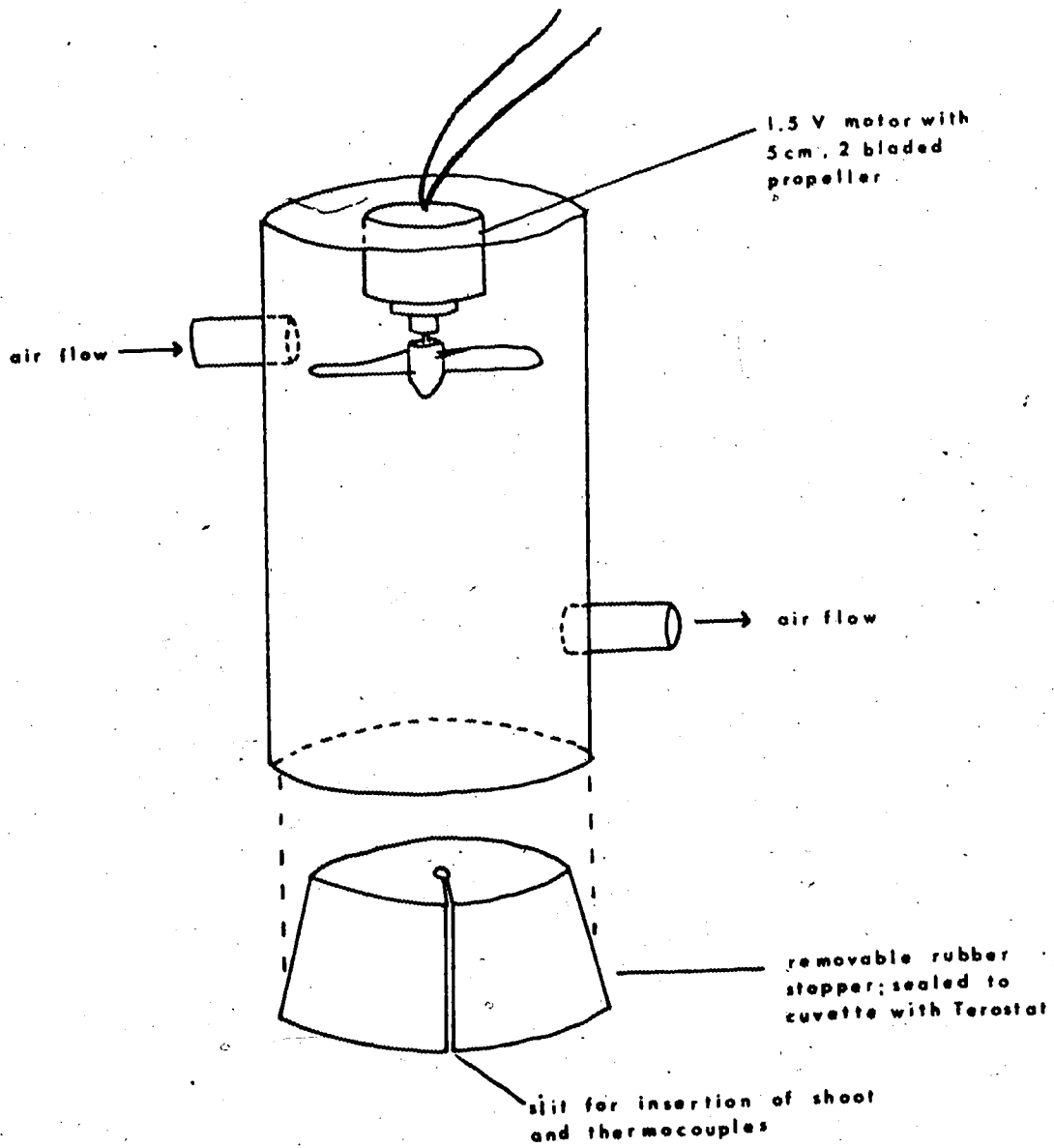
Fig. 28. Diagrams of Cu-constantan thermocouples.

Fig. 29. Diagram of cuvette used to measure the net photosynthesis of an intact leafy shoot of Ledum groenlandicum. (0.5X actual size)
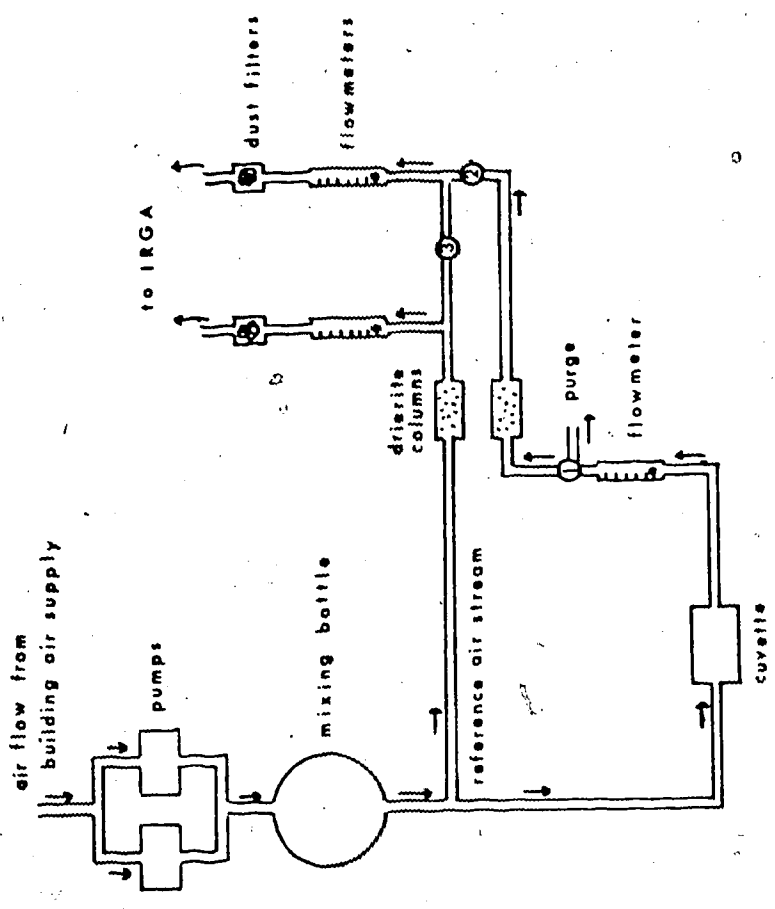
Fig. 30. Diagram of the gas flow system used in the measurement of net photosynthesis of intact shoots. The shoot was sealed in the cuvette. During ambient / ambient air stream measurements, valve 3 was open, valve 2 closed, and the cuvette air stream was purged at 1. During cuvette / ambient air stream measurements, purge valve 1 was closed, valve 2 open, and valve 3 closed.

## APPENDIX C

ISCO spectroradiometer analysis of the light quality of
growth chamber lamps and a quartz iodide lamp used in the
controlled environment studies.

Table 12.  An ISCO spectroradiometer analysis of the
light quality of various sources of illumination used in
controlled environment studies.

| Wavelength (nm) | Intensity of Irradiance ($\mu W\ cm^{-2}\ nm^{-1}$) | |
| --- | --- | --- |
| | Growth chamber* lamps at 40 cm | Quartz iodide lamp at 30 cm |
| 380 | .02 | 2.5 |
| 400 | .18 | 13.2 |
| 425 | .68 | 18.6 |
| 450 | 1.4 | 23.3 |
| 475 | 2.0 | 27.9 |
| 500 | 2.9 | 31.7 |
| 525 | 3.8 | 37.7 |
| 550 | 8.6 | 54.7 |
| 575 | 18.0 | 63.6 |
| 600 | 17.6 | 68.8 |
| 625 | 12.0 | 72.0 |
| 650 | 7.6 | 75.0 |
| 675 | 4.9 | 78.1 |
| 700 | 3.9 | 85.7 |
| 725 | 4.0 | 87.4 |
| 750 | 4.8 | 96.0 |
| 800 | 5.1 | 106.3 |
| 850 | 4.2 | 109.3 |
| 900 | 2.3 | 104.8 |
| 950 | 2.3 | 107.1 |
| 1000 | 2.3 | 103.3 |
| 1050 | 2.5 | 106.4 |
| 1100 | 2.7 | 88.5 |
| 1150 | 2.2 | 86.7 |
| 1200 | 1.5 | 75.3 |
| 1250 | 3.0 | 67.6 |
| 1300 | 4.1 | 62.7 |
| 1350 | 2.5 | 59.5 |
| 1400 | 1.0 | 54.0 |
| 1450 | .6 | 47.6 |
| 1500 | 1.3 | 43.9 |
| 1550 | 2.0 | 39.7 |

*combined cool white fluorescent and 100 watt incandescent
lamps

# APPENDIX D

Graphs of the relationships between leaf length X width and
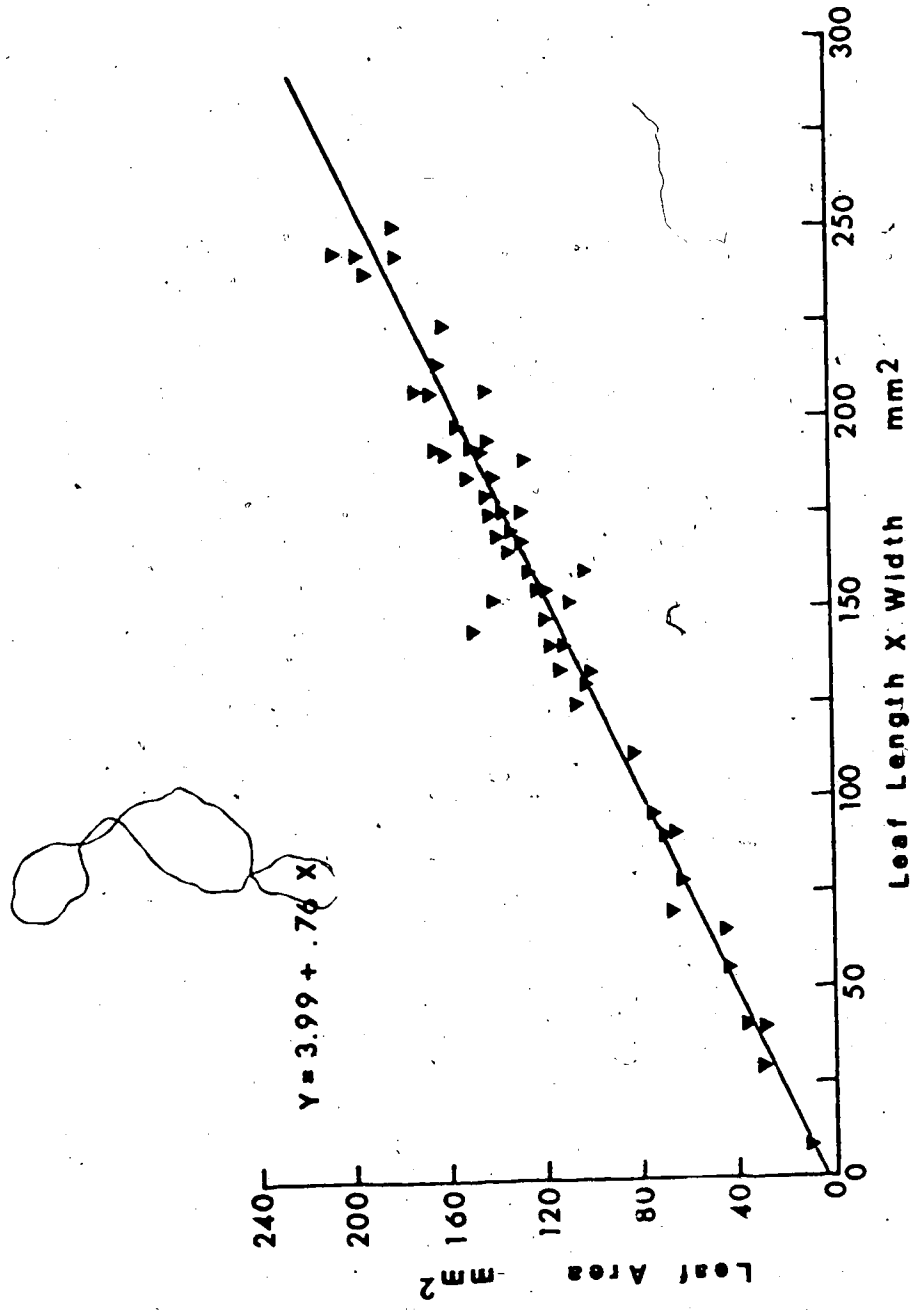
leaf area, and between leaf area and leaf dry wt.

Fig. 31. The relationship between leaf length x width and leaf area of *Ledum groenlandicum*. r2 = .9526.

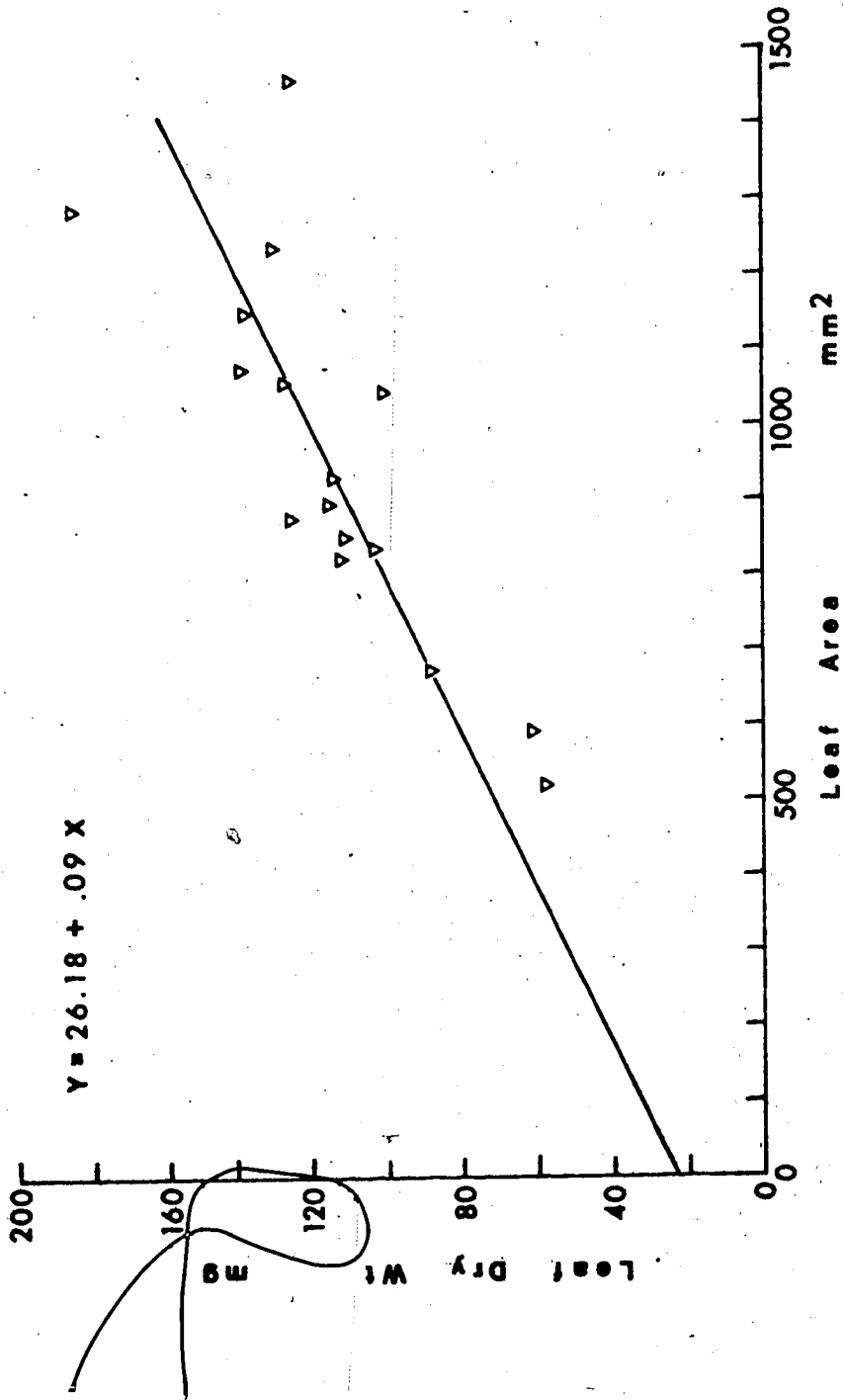Fig. 32. The relationship between leaf area and leaf dry wt of Ledum groenlandicum. $r2 = .6277$.

$Y = 26.18 + .09 X$

APPENDIX E


Experimental procedures and results pertaining to the

isolation of abscisic acid from <u>Ledum</u> <u>groenlandicum</u> leaves.

## MATERIALS AND METHODS

### Plant materials

A trial extraction for ABA was performed on the leaf tissue of the scarlet runner bean, _Phaseolus coccineus_. Seeds were sown in moist vermiculite and maintained under a 16 h photoperiod, a light intensity of 1500 ft-c, at 15 to 20 C, a R.H. of 50%, for 14 days. Water was withheld for four days, and by the eighteenth day the plants were noticeably wilted and were harvested. Leaf tissue was immediately frozen in liquid $N_2$ and stored at -25 C.

Collection of _Ledum groenlandicum_ leaves was made from plants growing under a _Populus tremuloides_ canopy at the University of Alberta Devonian Botanic Gardens, located 25 km SW of Edmonton. Approximately 100 fresh wt of leaves were harvested from the twigs in the field, and were immediately frozen in liquid $N_2$. On return to the laboratory, leaves of the first collection date were lyophilized. There is the danger of the production of endogenous ABA during an innefficient lyophilization, therefore, future collections were transferred directly to storage at -25 C. Fresh wt determinations in the field, prior to freezing, were not made, therefore, fresh wt refers to the weight of the frozen material prior to extraction.

## Extraction

Extraction of plant material was after a combination of methods similar to Davis, Heinz, and Addicott (1968) and Lenton, Perry, and Saunders (1971). All organic solvents used were of Baker 'Instra-Analyzed' Grade (J.T. Baker Chemical Co., Phillipsburg, N.J.). One hundred grams fresh wt of leaves were immersed in 400 ml ice cold 80% acetone. Sodium bicarbonate (5 g liter$^{-1}$) was added to the extraction medium to maintain a neutral or slightly alkaline pH that reduced the risk of isomerization and dehydration of ABA (Lenton et al. 1971). During all procedures, the exposure of the extract to direct sunlight was avoided to further reduce the risk of isomerization of 2-cis ABA to 2-trans ABA. The leaf tissue was chilled and infiltrated with the extraction medium, then was macerated in a Wareing blendor for 2 min. The homogenate was stirred overnight in the dark at 5 C, then the solid residue was removed by filtration.

The acetone extract was reduced to the aqueous phase on a rotary evaporator in vacuo at 35 C. The aqueous solution was frozen, thawed, and centrifuged at 16,000 xg for 1.5 h at 2 C. The suspended material was removed and was combined with the solid residue obtained after filtration for dry wt determinations. The residue was dried for 48 h at 70 C, and the dry wt was recorded.

The pH of the supernatant was adjusted to 2 with 2 N hydrochloric acid, and then was extracted three times with

equal volumes of ethyl acetate. The acidic ethyl acetate was combined and extracted four times with, alternately, one quarter its volume of 5% sodium bicarbonate solution, and one quarter its volume of deionized water. The combined aqueous extracts were adjusted to a pH of 2 and extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate then reduced to dryness on a rotary evaporator.

## Column Chromatography

The acidic fraction was purified on a Darco G-60 charcoal (J.T. Baker Chemical Co., Phillipsburg, N.J.)-diatomaceous earth (Grade III, Sigma Chemical Co.,St. Louis, Mo.)(1:2, w/w) column (diameter 2 cm); 1 g of diatomaceous earth being used for every 10 g of fresh plant material extracted. The acidic fraction was dissolved in 1 ml of deionized water and was applied to the top of the column. The column was rinsed successively with 50 ml of deionized water, and 50 ml of 30% aqueous acetone. The active material retained on the column was eluted with 250 ml of 60% aqueous acetone. The eluted acetone was reduced to the aqueous phase on a rotary evaporator. The aqueous phase was adjusted to pH 2 and extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate then

reduced to dryness on a rotary evaporator.

## Gas-liquid Chromatography

The dried plant extract was dissolved in 0.2 ml BSA (N,O,-bis- (trimethylsilyl)-acetamide)(Grade I, Sigma Chemical Co., St Louis, Mo.). The covered extract was allowed to stand for 30 min at room temperature before its contents were analyzed by GLC.

Chromatographic results were obtained with a Beckman GC-5 Chromatograph in conjunction with a Beckman 10" Recorder (Beckman Instrument Inc., Fullerton, Calif.). The chromatograph was fitted with dual 1.5 m x 3 mm stainless steel coiled columns, and was equipped with a flame ionization detector and a linear temperature programmer. A nitrogen carrier gas flow rate of 25 ml min$^{-1}$, a hydrogen flow rate of 25 ml min$^{-1}$, and an air flow rate of 300 ml min$^{-1}$ were maintained. Flow rates were calibrated with a soap bubble flowmeter. All gas supplies were of Linde Commercial Grade (Union Carbide Canada Ltd., Toronto, Ont.), and were fitted with dual stage pressure regulators. The nitrogen and hydrogen gas supplies were filtered through Nupro F-series 7 μ in-line filters (Nupro Co., Cleveland,Ohio) before entering the gas chromatograph. The columns were preconditioined overnight at 250 C prior to every major use. The column packing consisted of 5% QF-1 coated on 60/80 mesh, acid washed, DMCS treated Chromosorb W

(Chromatographic Specialties Ltd., Brockville, Ont.).

Chromatography consisted of injecting 3 ul of the BSA silylated extract into an isothermal column at 80 C; with the injector temperature at 190 C, the detector feed-through-line temperature at 180 C, and the detector temperature at 190 C. The column was kept at 80 C for 6 min, prior to a linear temperature increase of 15 C min$^{-1}$ for 8 min, reaching a maximum temperature of 200 C. The chroma███████of plant extracts were compared with those of a s███████ (±)-cis-trans ABA-trimethylsilyl (TMS) der█████████ug per 3 μl) (Grade IV, Sigma Chemical Co., St. Lou███ Mo.). An attenuation of 1 x 10⁴ was used, as a lower attenuation resulted in greater noise and baseline drift during the temperature programme.

## Ultraviolet Spectrophotometry

A 1 μl sample of the trimethylsilylated plant extract was dissolved in 0.005 N ethanolic sulfuric acid. The ultraviolet absorption spectrum, from 220 to 320 mu, was determined using a Unicam SP 1800 Ultraviolet Spectrophotometer (Pye Unicam Ltd., Cambridge, Eng.) set at maximum sensitivity. The ultraviolet spectrum was compared with that obtained from a standard 10 μg ml$^{-1}$ ABA solution having an absorption maximum at 262 mu.

# EXPERIMENTAL RESULTS

## Chromatography of Standard ABA

The first step in the detection of ABA was to standardize the peak height and retention time of a synthetic ABA-TMS derivative. A 15 µg per 3 µl ABA-TMS sample was used, and several temperature programmes were tried. Maintaining an isothermal column temperature was inadequate, as a column temperature of 180 C and above resulted in the ABA being masked by the solvent peak. Isothermal temperatures below 180 C gave longer retention times of the ABA-TMS derivative, but peaks became very diffused and tailed. A temperature programme similar to that used by Davis et al. (1968); starting with isothermal conditions at 60 C, and a linear temperature increase of 10 C min⁻¹ to 220 C was unsatisfactory. The slow temperature increase resulted in peak diffusion and tailing. Much higher rates of the temperature rise (30 to 40 C min⁻¹) gave the best peak resolution for the ABA standard, however, 15 C min⁻¹ was finally used to ensure adequate peak separation during the chromatography of the relatively impure plant extracts. Using the temperature programme described, the retention time of the standard ABA-TMS derivative was 11.2 ± 0.2 min (SD) (Fig. 33 a).
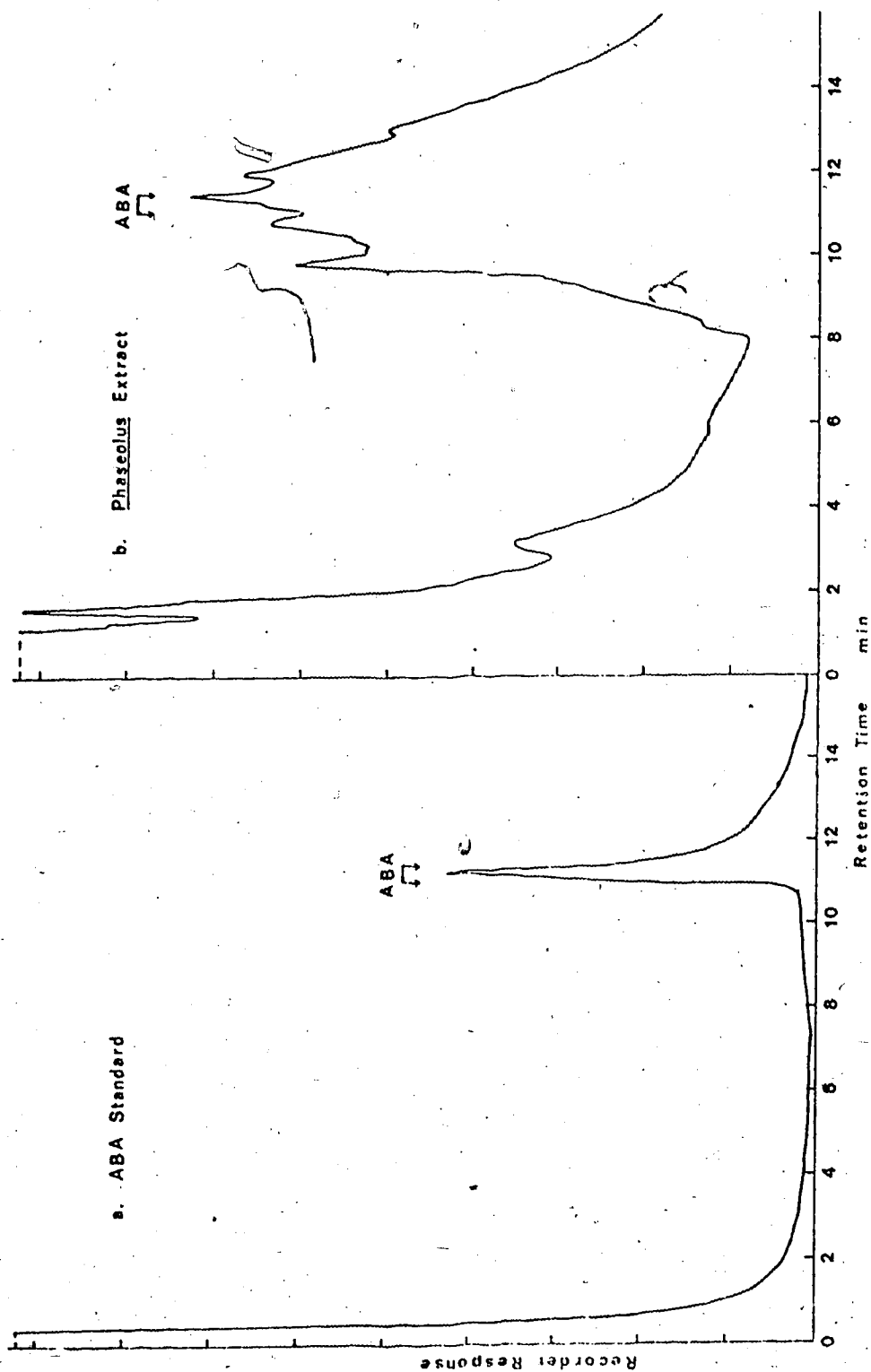
Fig. 33. Gas chromatograms obtained from the injection of 3µl of silylated a) synthetic ± cis abscisic acid and b) the acidic fraction of a Phaseolus vulgaris leaf extract. The linear temperature programme started at 6 min and was at a rate of 15 C per min from 80 C to 200 C. Attenuation was 1 x 10⁴. Arrows indicate the retention time of the standard ABA.

Ideally, one can determine the ABA concentration in an extract by calibrating the peak height against standards of known concentrations. This was not possible in the present study. Peak height and shape varied dramatically from run to run. Peak area determinations were also impractical because of the variable baseline drift. Determination of the peak areas of the chromatograms of plant extracts was impossible due to the peaks of interest being masked or being shoulders of larger peaks.

The results of the present study, therefore, were only qualitative. Even the qualitative occurrence of ABA in the plant extracts was questionable, because no chemical and biological assays were used to confirm the peak identities.

## Chromatography of the Phaseolus Extract

The Phaseolus coccineus plants were wilted prior to harvesting, therefore, high ABA levels were expected. Fig. 33 b shows that several large peaks occurred between the retention times of 10 to 13 min. One peak (indicated by the arrows) at 11.4 min corresponded to the retention time of the synthetic ABA. If a rough estimate of the ABA concentration in the bean extract was 15 $\mu$g per 3 $\mu$l, then the concentration of ABA in the wilted Phaseolus leaves was 5 $\mu$g g$^{-1}$ fresh wt, or 51.6 $\mu$g g$^{-1}$ dry wt (only 0.1 ml BSA was used for the bean extract). Raschke and Zeevart (1976) found ABA concentrations of 3.9 $\mu$g g$^{-1}$ fresh wt and 22.7 $\mu$g

g$^{-1}$ dry wt in young wilted leaves of _Xanthium strumarium_. The values found in the present study were gross estimates and were calculated for curiosity sake. However, the values calculated here and by Raschke and Zeevart (1976) were orders of magnitude higher than other literature values. Due to the lack of a critical bioassay, and the poor resolution of the bean extract chromatogram peaks; neither qualitative, nor quantitative assumptions can validly be made about the occurrence of ABA in _Phaseolus_.

Chromatography of _Ledum groenlandicum_ Extracts

Chromatograms of _Ledum groenlandicum_ extracts (Figs. 34 a to e) provide no information as to the presence of endogenous abscisic acid because there were no obvious peaks corresponding to the retention times of the ABA standard. There were faint suggestions of ABA-like peaks in the chromatograms of September 12 and of January 20 (Figs. 34 b and e), however, the peak of September 12 had a fast retention time of 10.9 min. The results for the occurrence of ABA in _Ledum groenlandicum_ were, therefore, inconclusive. If ABA was in the _Ledum_ extracts, it was present in undetectable amounts and masked by other impurities.

The percent water content, on a dry wt basis, was calculated from the fresh and dry wt determinations of leaf tissue. There was a decline in the leaf water content accompanying a drop in leaf water potential and an increase
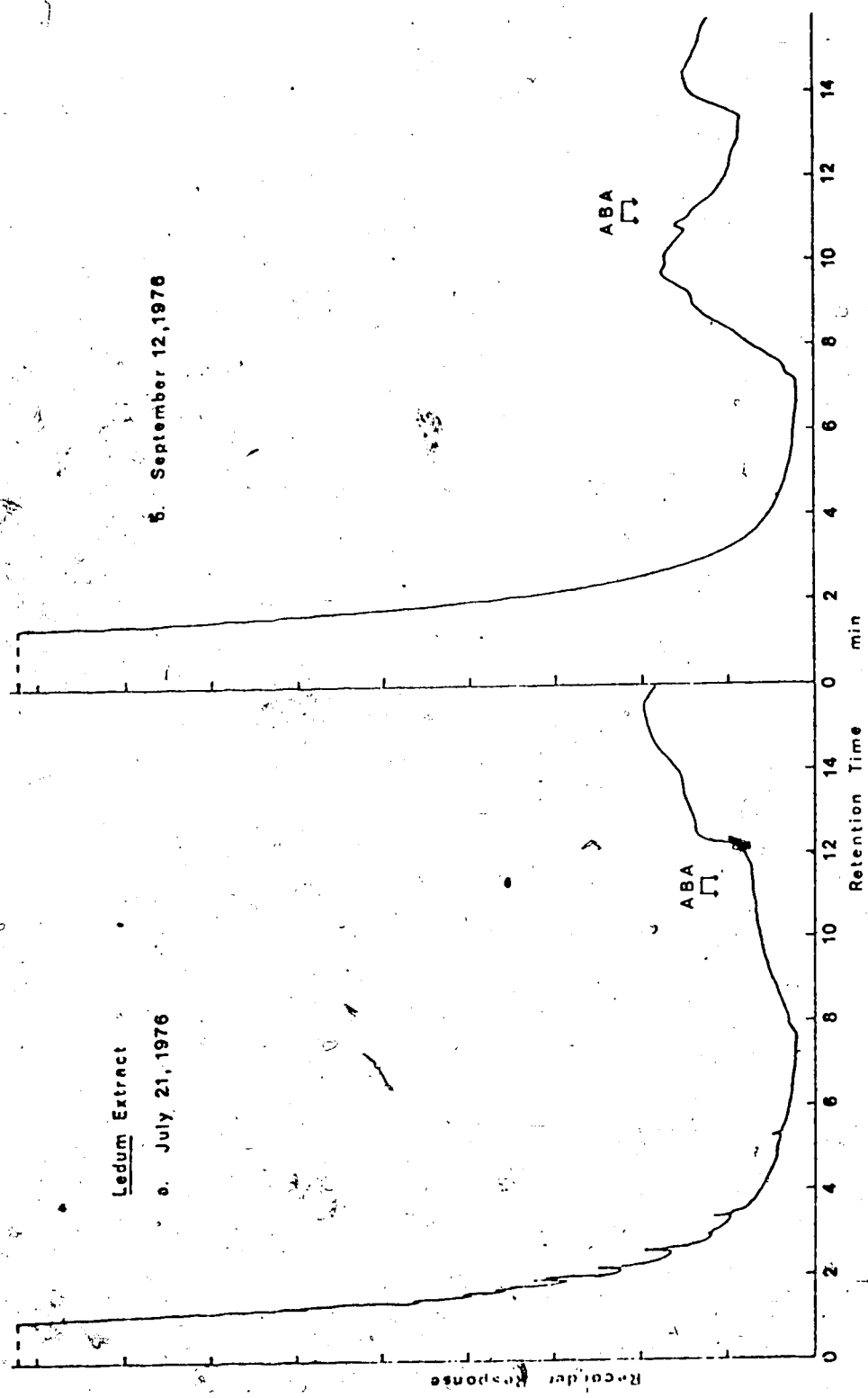
Fig. 34. Gas chromatograms obtained from the injection of 3 μl of the silylated acid fraction of _Ledum groenlandicum_ leaf extracts. A linear temperature programme started at 6 min and was at a rate of 15 C per min from 80 C to 200 C. Attenuation was 1 X 10⁴. Arrows indicate the retention time of standard ABA. Dates in Fig. 34. a) to e) refer to the sample collection date.

c. October 9, 1976

d. November 8, 1976

ABA

ABA

Recorder Response
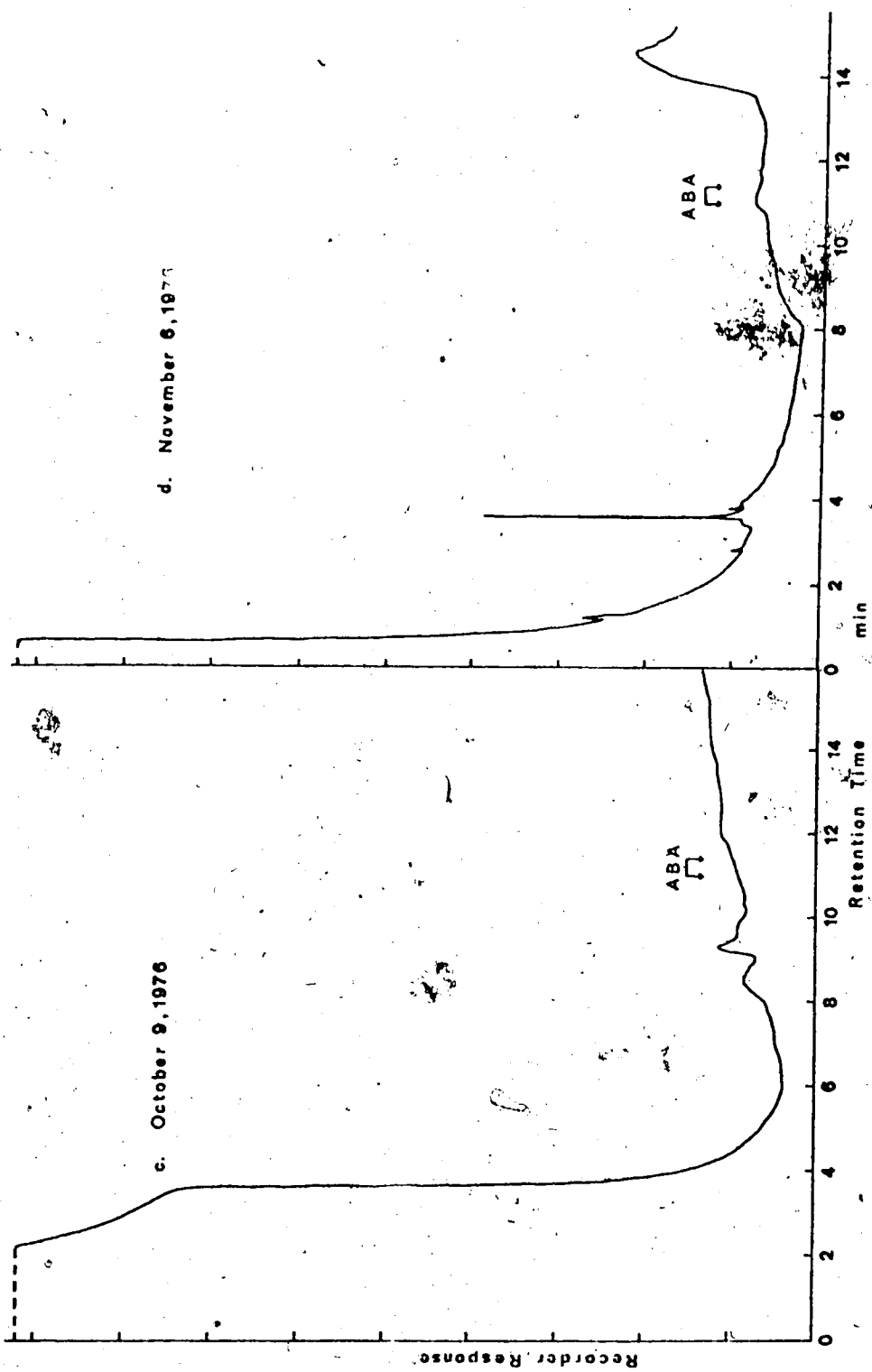
Retention Time

min

Fig. 34. For explanation of graphs refer to preceding page.
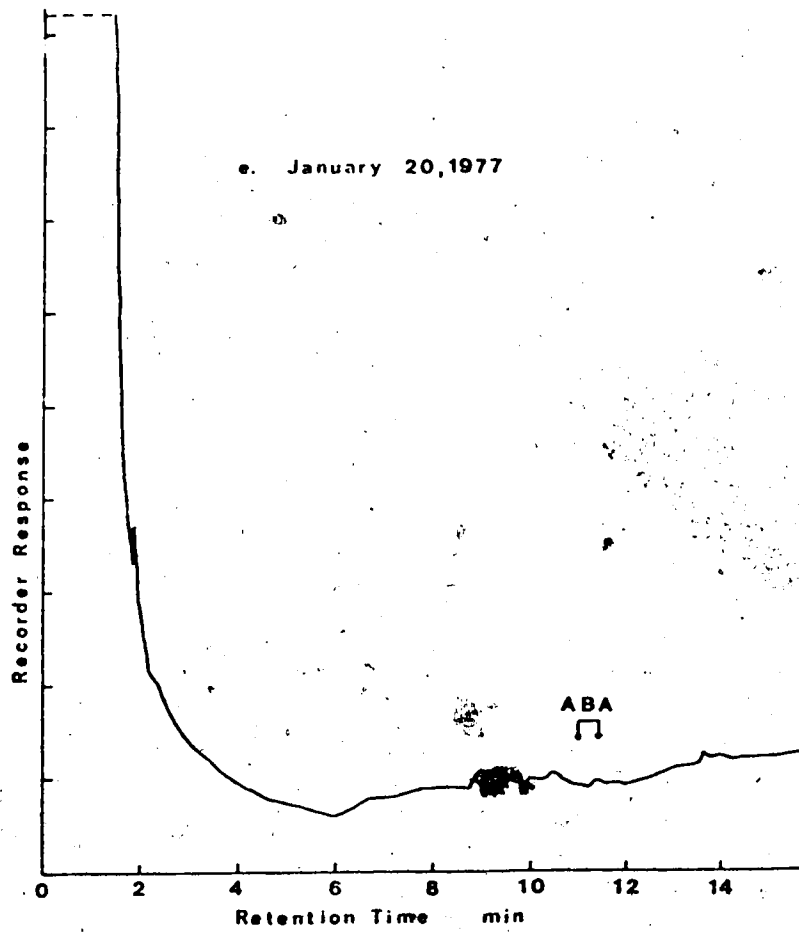
Fig. 34.  For explanation of graph refer back 2 pages.

in tissue hardiness. Lower leaf water potentials were, therefore, partially due to the dehydration of leaf tissue.

## Ultraviolet Spectrophotometry

An attempt to detect ABA using ultraviolet spectrophotometry was made. Ultraviolet spectrophotometry is considered to be a less sensitive method of ABA detection than gas-liquid chromatography. Fig. 35 a shows the ultraviolet spectrum for a standard ABA solution, having an absorption maximum at 262 mu. Figs. 35 b and c show the ultraviolet spectra for the Phaseolus extract and the Ledum groenlandicum extract of July 12, respectively. These spectra showed the bimodal absorption maxima typically found in tissue extracts rich in flavinoids. The Phaseolus extract contained a flavinoid similar to caffeic acid, and the Ledum groenlandicum extract contained a flavinoid of unknown identity (Deaford personal communication). To remove the flavinoids from the plant extracts would have required extensive purification using thin layer chromatography and column chromatography. This was not feasible in the present study due to the shortage of plant material and time. Ultraviolet spectrophotometry, therefore, was not useful in the detection of ABA due to the impurities in the plant extracts masking the zone of the ABA absorption maximum.
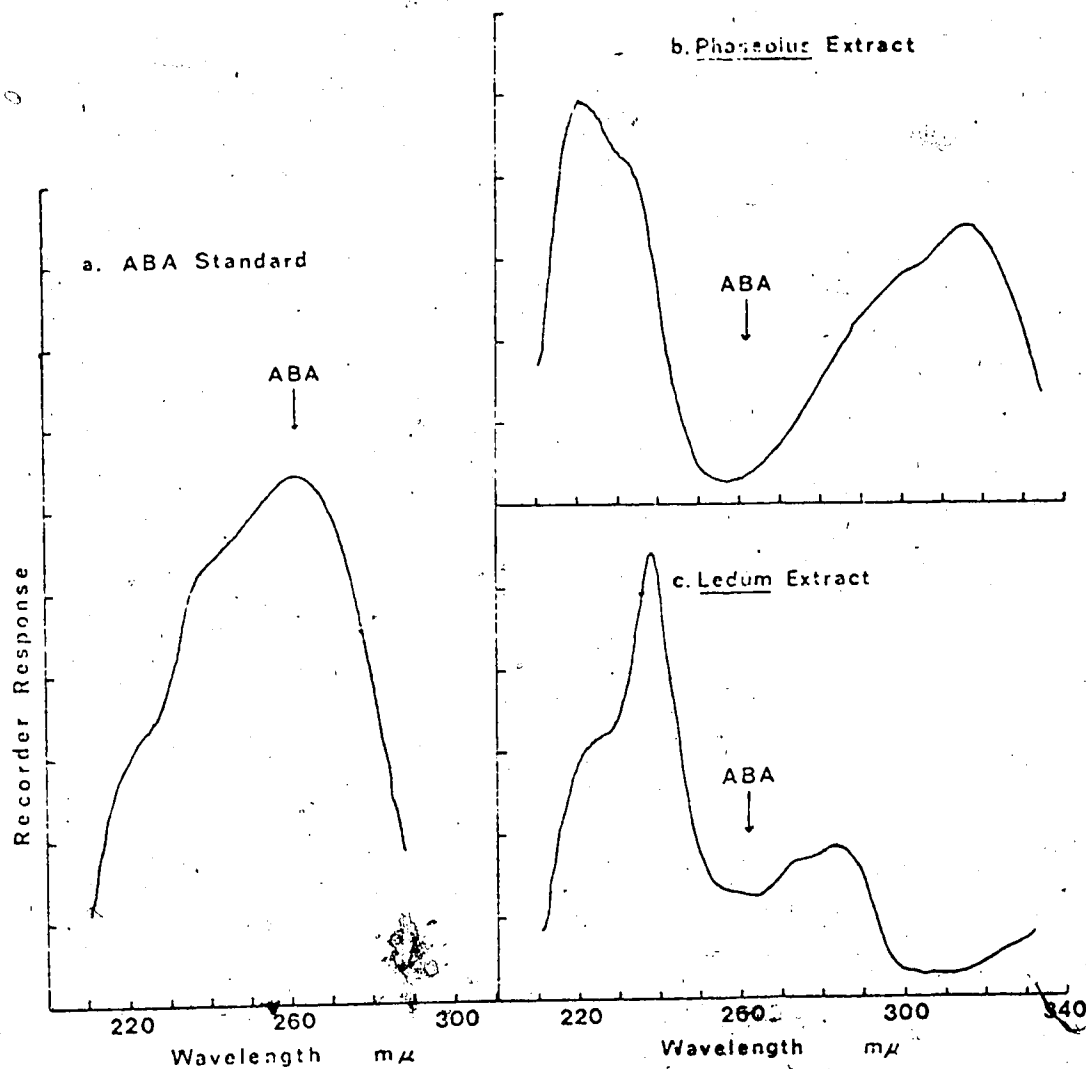
Fig. 35. The ultra violet spectra of silylated extracts dissolved in .005 N ethanolic sulfuric acid of a) synthetic + cis abscisic acid, b) Phaseolus vulgaris, and c) Ledum groenlandicum. Arrows indicate the absorption maximum for standard + cis abscisic acid at 260 mu.