STUDIA FORESTALIA SUECICA

Nr 77 1970

Frost hardiness in Scots pine (*Pinus silvestris L.*)

I. Conditions for test on hardy plant tissues and for evaluation of injuries by conductivity measurements

Frosthärdighet hos tall (Pinus silvestris L.)

I. Metoder för testning av härdigt plantmaterial och för bestämning av skador med ledningsförmågemätningar

by

ARON ARONSSON and LENNART ELIASSON

Department of Plant Ecology and Forest Soils

SKOGSHÖGSKOLAN
ROYAL COLLEGE OF FORESTRY
STOCKHOLM

ABSTRACT

A simple routine procedure for testing frost hardiness of woody species has been worked out, using *Pinus silvestris* (L.) as the experimental plant. Shoots or twigs are placed in deep-freezes maintained at different temperatures. The rate of cooling of the plant tissues depends on the temperature of the deep-freeze. It is more rapid than the rates usually employed in freezing tests. The merits and disadvantages of this freezing method are discussed. The degree of injury is determined by a modification of the conductivity method. The procedure has been used for determining changes in frost hardiness of Scots pine from October to May. The values obtained are reproducible, and even small changes in degree of hardiness of very hardy plant tissues may be determined by the method.

Ms. received 5 December 1969

ESSELTE TRYCK, STHLM 70

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Introduction

Injuries to plants due to low temperature are a practical problem of great importance. The interest in this field on the part of agronomists, horticulturists and plant breeders is of long standing, as is shown by the review by LEVITT (1956). The literature on frost hardiness in woody plants has been reviewed by PARKER (1963). While the hardiness of fruit trees has been intensely investigated, there has been surprisingly little research into this property of forest trees, in spite of severe damage often caused to forest tree species in winter.

In northern Sweden (Norrland), damage caused in hard winters and by occasional summer frosts may seriously impair the development of young pine and spruce. EICHE (1966) presented a large observational material, obtained from provenance trials in Norrland, on cold injuries to Scots pine. Field observations reported by STEFANSSON & SINKO (1967) show that climatic stress is an important cause of plant death in plantations on abandoned fields. ANDERSSON (1968) determined temperature variation and frost damage to coniferous regrowth on areas liable to be affected by summer frosts. He concluded that frost is a very common cause of plant death on many sites in Norrland which are difficult to reafforest. BÄRRING (1967) described injuries to planted Norway spruce seedlings which were apparently due to infavorable weather during late winter or early spring.

As has been pointed out by TAMM (1966), field survival in relation to winter weather involves complex interactions between several factors. In the observation of damage in the field it is often difficult to assess the part played by low temperature on the one hand and by other factors, such as drought and parasitic microorganisms, on the other. The only way to come to a better understanding of this problem is to study frost hardiness of conifers under better controlled conditions.

The nature of the injuries caused to plant cells by freezing has been the subject of much research. Recent reviews in this field are presented by LEVITT (1966 a), OLIEN (1967), and MAZUR (1969). It has been shown that it is not the low temperatures as such that kill the tissue. The cause of injuries during freezing is the formation of ice crystals, either in the protoplasm (intracellularly) or between the cells in the intercellular spaces. If cooling and rewarming proceed very rapidly, ice crystal formation is prevented and in this case damage may be avoided (LUYET, 1951; SAKAI & YOSHIDA, 1967). Thin slices of tissue may thus be cooled to the temperature of liquid nitrogen (—196 °C) without being killed.

Intracellular formation of ice crystals will nearly always kill the tissue, probably as a result of mechanical injury to the protoplasm. In hardened plants, intracellular ice formation is prevented at moderate rates of cooling, probably because of the increased permeability of the plasma membrane to water. Owing to the increased permeability, ice formation in the intercellular spaces is facilitated. Another possibility is that the cell walls of hardy plants contain substances which interfere with ice crystal growth (OLIEN, 1965).

Ice formation in the intercellular spaces will cause dehydration of the protoplasm. In non-hardy cells proteins, especially membrane proteins, will be altered by this dehydration to such an extent that the cells die. Hardening involves biochemical changes in the cell which prevent destruction of essential proteins. Sugars which accumulate in the cells during hardening due to hydrolysis of starch or as a result of photosynthesis are considered to function as protective substances for the sensitive proteins. Recent results and hypotheses regarding the biochemistry of frost injuries and frost hardening are given by LEVITT (1966b, 1967), HEBER & ERNST (1967) and TUMANOV (1967).

The seasonal variation in frost hardiness, and factors of importance for the development of hardiness in woody plants, were discussed by PARKER (1963) in his review. The ability of the plant to withstand low temperatures without injury is lowest during the growing season. Organs which are growing rapidly are particularly sensitive. As has been shown by many investigations, frost hardiness increases during the autumn and reaches a maximum during the winter. Low temperature is an important factor in the development of hardiness and it is also a prerequisite for the maintenance of that hardiness. During late winter and spring there occurs for this reason a gradual decrease in hardiness. Besides temperature, other environmental factors, especially light and the supply of nutrients, influence hardiness. Hereditary factors are of great importance to the ability of the plant to develop hardiness. It has, for instance, long been known that different provenances of Scots pine show differences in frost hardiness (KIENITZ, 1922). LANGLET (1936) showed that these differences are connected with differences in water and sugar content. The time of sprouting and the course of growth of shoots and needles is of importance as regards susceptibility to frost during the spring and summer, as has been shown by ANDERSSON (1968) and other workers.

The present investigation was begun on the initiative of Professor C. O. TAMM in connection with other efforts at present being made at the Royal College of Forestry to elucidate the various factors which impede reafforestation, especially at high altitudes in Norrland. The aim of the work is to study the way in which climatic and nutritional factors influence frost hardiness

in various provenances of Scots pine. It is important in this work to be able to measure, accurately and reproducibly, the degree of hardiness during different phases of the development of the plants, in relation to different climatic conditions.

This paper deals primarily with the methodological problems in work of this kind. It is evident from the foregoing that frost hardiness is a dynamic and composite property of plant cells. It may be expressed in relative values, allowing comparisons to be made between the hardiness of different plant materials and of the same material on different occasions. The values obtained are, however, influenced by the methods used in their determination. For this reason it is necessary to evaluate critically the methods used. Some special requirements had to be considered in the present investigation. It is planned to carry out part of the work in the field. For this reason, only apparatus capable of being installed in a large caravan could be used. Furthermore, it was considered desirable to select an experimental routine which could enable one person to carry out the necessary tests. The high degree of hardiness developed by some woody species in cold climates (PARKER, 1963) presents some special problems of measurement. A convenient experimental routine for work with such very hardy conifers will be described and some results will be presented.

Survey of methods for determining frost hardiness

Most investigations of frost hardiness are based on the following two phases:

1. *Exposure of the plant material to low test temperatures*
2. *Determination of the effect of the cold treatment*

Less direct methods, in which properties of the tissue showing a more or less close correlation with frost hardiness are determined, have not been found reliable (LEVITT, 1956; 1966a).

The least sophisticated method is to observe the response of plants in the field to naturally occurring low temperatures. The plants to be tested are often planted in regions having severe winters and plant mortality and local damage are recorded. Results obtained in such experiments are often difficult to interpret, owing to the many complex relationships involved (OLIEN, 1967). The fact that visible symptoms usually develop slowly in injured woody plants makes it difficult to relate observed injuries to periods of low temperature. The fortuitous variation in winter minimum temperatures also restricts experimentation. Artificial freezing in the field is sometimes used (GLERUM *et al.*, 1966; READ, 1967), but for technical reasons it is difficult to carry out such treatments under well defined conditions.

For freezing experiments under controlled conditions it is necessary to work in a laboratory furnished with freezing equipment. Artificial freezing as a test for frost hardiness was first used as a tool for comparing different varieties for agricultural crop plants (ÅKERMAN, 1927). Generally, good agreement is obtained between hardiness determined in laboratory tests and survival in the field (LEVITT, 1956). In the case of woody plants, the test is usually made on only part of the plant. For this reason it is possible to study trees growing in different habitats after transfer of twigs to the laboratory.

Several factors are of importance in a freezing test. Most attention has been devoted to *the lowest temperature* a plant can endure without damage. As was demonstrated for conifers by PFEIFFER (1933) *the rate of cooling* will influence this temperature. *The length of time* for which the plant material is maintained at the low temperature is also of importance, as was pointed out by DAY & PEACE (1937). Also *the rate of thawing* may influence the development of damage (ILJIN, 1934). Hence to make the results from different tests comparable, it is important that the course of cooling and rewarming, as well as the duration of the test, is the same for all tests. The most common way of determining frost hardiness is to lower the tempera-

ture of the material at a standard rate, often 1—5°C per hour, to a series of predetermined temperatures. The "frost killing temperatures" determined in this way are of course only relative values. They are not identical with the temperatures which will kill the plants in nature, where other and very varying rates of temperature change occur. The conditions for the freezing procedure used in different laboratories vary within wide limits and the frost killing temperatures determined are not generally comparable.

There are several established methods for determining the damage caused to the tissues during the freezing test. The most important may be grouped as follows:

1. *Observational methods.* Symptoms of injury, such as discoloration or wilting, are observed either directly after thawing or after keeping the plant material in a green-house for some time. Usually only an ocular inspection of the plant sample is made, sometimes after sectioning for examination of cambium and wood. The method may also include closer examination of the tissue under the microscope. Observational methods have been extensively used in investigations of frost hardiness in agricultural crop plants (Åkerman, 1927, 1949) and in fruit trees (Granhall & Oldén, 1950; Oldén, 1955, 1957). Also many investigations of hardiness in forest trees rely on such methods for evaluating the extent of injury (e.g. Tranquillini, 1963; Day & Barrett, 1962).

2. *Plasmolysis test.* If the cells are killed, they lose their ability to be plasmolysed or deplasmolysed (Siminovitch & Briggs, 1953). This test may be used to decide whether the cells of a tissue are dead or alive after freezing.

3. *Staining tests.* Staining may be by means of dyes, for instance neutral red or acridin orange, which penetrate living and dead cells to different extents (vital staining). Another type of staining depends on the metabolic activity of the cells. Triphenyltetrazolium chloride is a colourless substance which is reduced to red formazan in living cells but not in dead. This substance has been used to test viability in woody plants after freezing, as reviewed by Parker (1963). If the red pigment is extracted and determined by colorimetric methods, quantitative values may be obtained (Steponkus & Lanphear, 1967).

4. *Measurement of electrical conductivity.* This method gives a measure of the injury caused to the tissue, because dead or injured cells leak salts. Conductivity is a measure of the quantity of electrolytes in an aqueous solution. It is possible to make measurements directly in the plant tissue (Henze, 1967; Wilner, 1961, 1967; Glerum, 1969). A more convenient method is to place sections of the plant in pure water after the freezing test and measure the conductivity of the solution after a standard time. This method, often

referred to as the exosmotic method, was introduced into research on frost hardiness by DEXTER *et al.* (1930, 1932). It has been extensively used in investigations on frost hardiness in fruit trees (STUART, 1939; VILNER, 1960, 1962; TAMAS, 1961, 1963; NYBOM *et al.*, 1962 and others). In investigations on coniferous species it has been used by, amongst others, MCGUIRE & FLINT (1962), CARPENTER *et al.* (1963) and ZEHNDER & LANPHEAR (1966). SIMINOWITCH *et al.* (1964) determined the release of amino acids from the tissue instead of that of electrolytes and used this as a measure of injury caused by freezing.

Methods for determining whether a cell or tissue is alive or dead have been more fully treated by PARKER (1953). A method for rapidly determining the killing effect of freezing was recently published by McLeester *et al.* (1969). It depends on the fact that the freezing curves are different in living and dead tissues.

Rapid freezing test method

Test conditions

The freezing tests were carried out in deep-freezes (size 225 l). In order to obtain rapid and reproducible courses of cooling, only a relatively small quantity of material was placed in each deep-freeze. The deep-freezes were cooled initially to the selected minimum temperatures. The rate of cooling with this technique thus depends on the initial and the final temperatures. A lower minimum temperature means a greater stress on the plant material, not only because of the lower temperature *per se*, but also because the rate of cooling will be greater.

Both observational methods and measurements of electrical conductivity were used to determine the degree of injury caused by freezing. Because of the objective character of the conductivity method and the possibility of obtaining reproducible values rapidly by this method, it was chosen as a routine method for the experiments.

The material consisted of shoots of one or two year old pine seedlings grown in a plastic green-house or of the previous summer's growth from twigs of 15—20 year old pines. The following procedure was routine in most of the work. On collection the shoots were immediately sealed into polythene bags and taken to the laboratory. The time schedule for the treatments is shown in Figure 1. In order to give the material the same initial temperature on each occasion of testing, the closed plastic bags were stored in a refrigerator at 4°C for 18—19 hours before the freezing test was carried out. A standard pretreatment is necessary because the rate of freezing is dependent on the initial temperature of the plant material. An objection to this procedure would be that some dehardening or hardening occurs during this temperature equilibration in material which in the field is subjected to temperatures substantially different from the refrigerator temperature. This effect has to be taken into account, but it is evident that it will decrease rather than exaggerate differences between different materials. Possibly there will also be some "thawing effect" in materials brought in from low out-door temperature during winter.

For the freezing treatments, parallel samples of the material were placed in deep-freezes kept at the approximate temperatures —12°C, —22°C and —44°C, for six hours. Later, a deep-freeze set at —32°C was also employed. The control sample was left in the refrigerator at 4°C. The shoots or twigs were removed from the plastic bags during freezing and spread out in the deep-freezes. The air in these was circulated by fans both to make cooling

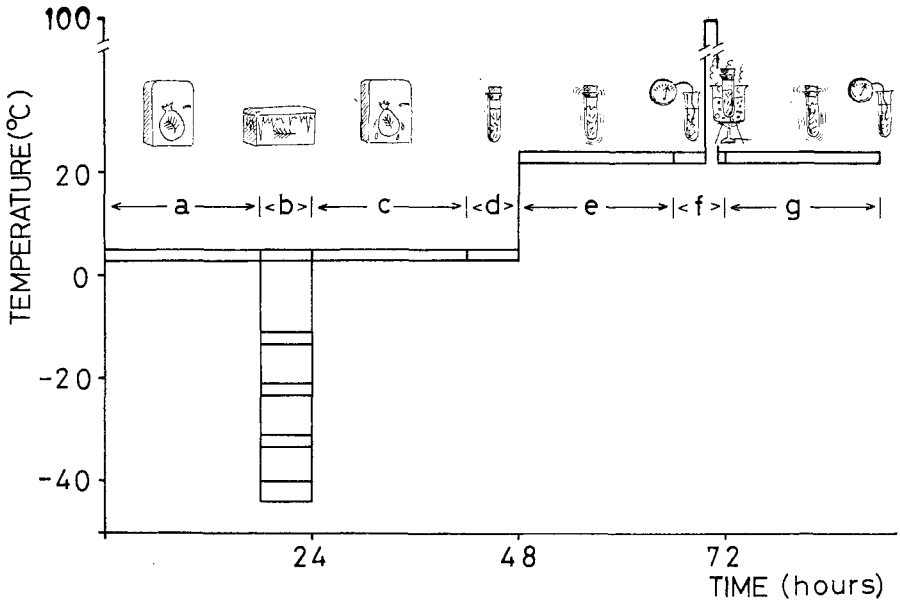

Fig. 1. Time schedule for the freezing test and conductivity determinations.
a. The plant material in refrigerator in closed plastic bags at 4°C for 18—19 hours.
b. Freezing treatments at different temperatures for 6 hours. The shoots spread out in the deep-freezes.
c. Thawing in refrigerator at 4°C for 18—24 hours in closed plastic bags.
d. Preparation of the material; soaking in distilled water in stoppered test tubes.
e. Shaking of the tubes for 18—20 hours at room temperature.
f. Measurement of conductivity at 25°C. Boiling.
g. Shaking for a new period of 18—20 hours followed by a second measurement of conductivity.

uniform and reproducible and to counteract supercooling of the tissues. The courses of temperature lowering in stems of 4 mm diameter are shown in Figure 2. The temperature was followed by means of thermistors placed in 5 mm deep and 2 mm wide holes in the middle of the stem base of twigs from ten year old pines. The rate of cooling is, of course, dependent on the thickness of the organs. In the needles it is higher than in the stems and buds. The material was thawed in the refrigerator for 18—24 hours. During this time the shoots were kept in closed plastic bags to prevent evaporation and translocation of electrolytes within the shoots.

Before determination of conductivity, sections of stems or needles or whole apical buds were placed in distilled water in stoppered test tubes (25 × 100 mm) at room temperature (20—23 °C). The needles were cut out in 8 mm, the stems in 5 mm long sections. The amount of water used was 20 times the fresh weight of the tissue. Usually 0.5 g fresh weight of tissue was

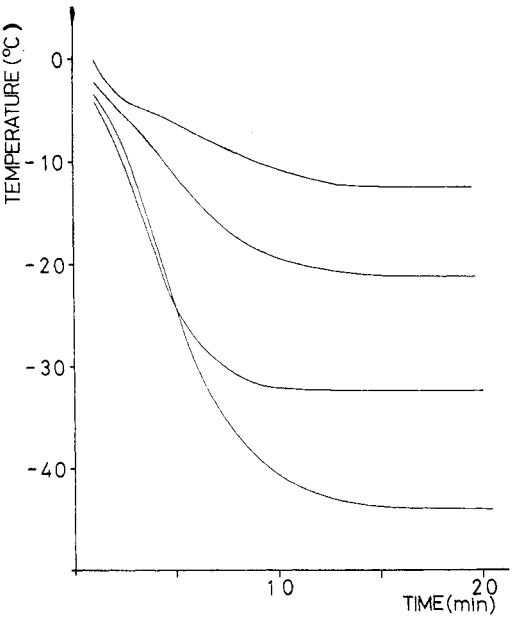

Fig. 2. Time course of temperature lowering in the deep-freezes used. These had attained the minimum temperature at the start of the experiment, while the plant material was transferred from +4°C. Temperature registered every minute by thermistors placed in the middle of stems of 4 mm diameter. Mean values for 8—10 determinations.

placed in 10 ml water. For sections of needles and stems, three or four determinations were made on each sample, while the buds in each sample sufficed for only one test tube. The tubes were placed on a shaking table and shaken for 18—20 hours. The extraction time was chosen on the basis of a determination of the time course of the release of electrolytes (Figure 3). Bacterial contamination progressed rapidly after 24 hours and is probably at least partly the cause of the increased leakage of electrolytes after prolonged soaking of the sections in water. For stem sections the course of electrolyte release was somewhat faster.

Because electrical conductance is highly temperature-dependent, the temperature of the test tubes was adjusted to 25°C on a thermostat bath before measurement of the conductivity. Measurements were made by dipping the conductivity cell directly into the test tube without removal of the tissue. The instrument used was Philips' direct-indicating measuring bridge PR 9501. The measurements were made at the frequency of 80 Hz. After the first measurement the tissues were completely killed by placing the tubes in a boiling water bath for ten minutes. No increase in the release

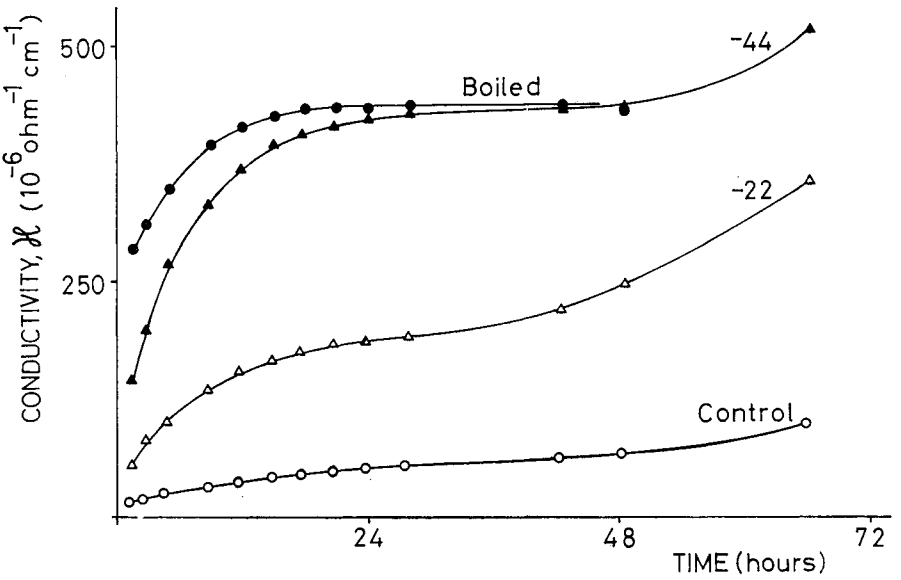

Fig. 3. Time course of increase in conductivity in 10 ml distilled water with 0.5 g needle sections (treated as indicated) added.

of electrolytes was obtained by longer boiling, as is shown by the values of Figure 4. Evaporation of water from the tubes was made negligible during boiling by leaving the stoppers in place, but loosened in order to prevent increased pressure in the tubes. The boiled samples were shaken for a further 18—20 hours, after which the conductivity was measured a second time.

The first value for conductivity ($\varkappa_{\text{frozen}}$) depends on the degree of injury to the tissues during the freezing treatment. The value obtained in the second measurement ($\varkappa_{\text{boiled}}$) is a measure of the electrolytes diffused out from the completely killed tissue. It should be stressed that the object of killing the tissue is not to determine the total amount of electrolytes but to obtain a reference value which shows the release of electrolytes from 100 per cent damaged tissue under conditions similar to those used after freezing and thawing. The conductivity values obtained for tissue severely injured during freezing should consequently be similar to that obtained for boiled tissue. There is a considerable variation in the conductivity values for different tissues, mainly because of variation in the content of electrolytes. For this reason a convenient measure of the degree of injury is the *relative conductivity* (RC) defined in the following way:

$$\text{RC}=\frac{\varkappa_{\text{frozen}}}{\varkappa_{\text{boiled}}}\cdot 100$$

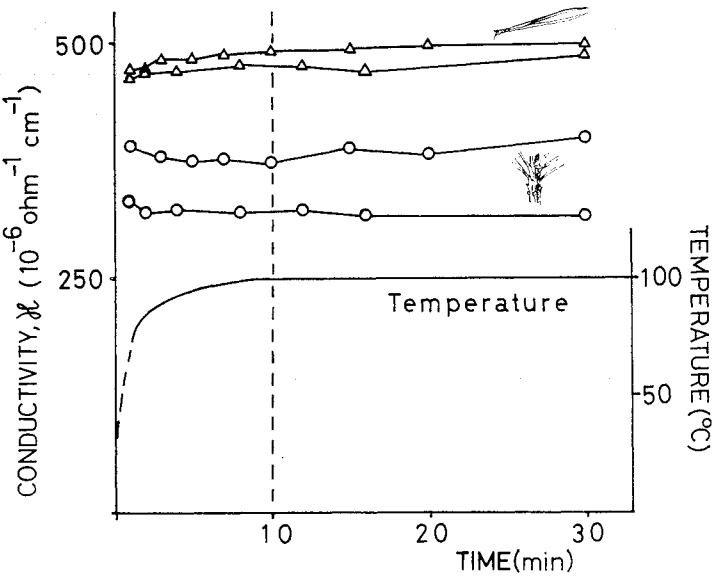

Fig. 4. Effect on the subsequent release of electrolytes of the time at which the tubes were placed on the boiling water bath. The temperature curve shows the course of increase in temperature in the solution in the test tubes. Symbols: △ needles; ○ stem sections. Two different experimental series for each material.

Similar relative or percentage values have been used by EMMERT & HOWLETT (1953), WILNER (1961), MCGUIRE & FLINT (1962) and others. These authors, however, stated that they used the total amount of electrolytes in the tissue as a reference value. As is pointed out by TAMAS (1961) and NYBOM *et al.* (1962), no complete diffusion of all electrolytes from the tissue sections is to be expected under the conditions used. This is neither necessary nor desirable, since the reference value should be of the same magnitude as the value obtained for tissue completely killed by freezing. For this reason there is no need to use procedures which increase the release of electrolytes as, for instance, changing the solutions before boiling or autoclaving, as proposed by CARPENTER *et al.* (1963). Other kinds of relative values have been used in frost hardiness research by NYBOM *et al.* (1962) and by SCHUBERT (1965).

The variation in conductivity values obtained for plant material used in the present investigation is illustrated by the examples in Table 1. It is evident that the conductivity values ($\varkappa_{\text{frozen}}$) are no good measure of the degree of injury if used directly. The values obtained at a certain degree of injury depend on the electrolyte concentration in the tissue and probably

Table 1. Examples of conductivity values obtained for different pine materials. The values were selected from samples giving *relative conductivity* (RC) values 41—44 after the freezing treatment. For details of the plant materials used see page 17.

Plant age years	Tissue	Conductivity value frozen	boiled	RC
1	Buds	280	642	44
1	Needles	250	564	44
1	Stems	240	582	41
2	Buds	194	462	42
2	Needles	249	578	43
2	Stems	152	365	42
20	Buds	209	485	43
20	Needles	177	405	44
20	Stems	148	353	42

also on factors such as section size, which may influence the rate at which the electrolytes are released from the injured tissue sections into the water. It is also evident that large errors in the conductivity values may be due to variations in the water content of the samples. From the RC values variation due to these causes is largely or completely eliminated.

Effect of rates of cooling and rewarming

In order to compare the freezing test used in the present work with freezing tests employing slower rates of cooling and rewarming, an experiment was carried out with different rates of temperature change. The degree of injury, as measured by the RC values, is given in Table 2. "Rapid" cooling and rewarming rates were obtained by the procedures normally employed in the freezing tests (see Figure 2). "Slow" cooling and rewarming rates

Table 2. Effect on Relative Conductivity of different rates of temperature decrease and increase, respectively, in the freezing test.[1]

Rate of cooling	rewarming	Relative Conductivity Needles	Stems
Slow	Slow	14	20
Slow	Rapid	30	27
Rapid	Slow	80	71
Rapid	Rapid	54	56
Control values		9	15

[1] Parallel samples of hardy pine twigs collected on 27 Feb. 1968 and stored at —12°C for three weeks were used. After thawing for 27—28 hours at 4°C the twigs were cooled to —44°C at a *slow* (5°C per hour) or *rapid* (by placing the twigs directly in the cooled deep-freeze, see Fig. 2) rate. Twigs were rewarmed after six hours at a *slow* (6—7°C per hour) or *rapid* (by transferring the twigs to 4°C in refrigerator) rate.

were obtained by manual operation of the deep-freezes. The twigs were in this case enclosed in insulated boxes of paper which were moved between the deep-freezes. The temperature in the paper boxes was followed by means of thermistors. The material used in this experiment was twigs removed from the middle of the crown of one 20 year old pine.

As is shown by the values in Table 2, only little injury was caused to the tissues at slow cooling rates. Rapid cooling combined with the usual rapid rate of thawing, on the other hand, caused definite injury. The highest degree of injury was obtained when rapid cooling was combined with slow rewarming. The reason for the greater injury at a slow rate of rewarming in this case is probably that the tissue is held at low temperatures for a longer period with this treatment. Crystal initials formed in the protoplasm during the rapid freezing will have longer to grow to a size which will damage the cells (cf. LUYET, 1967). This experiment shows that the rapid cooling rate used in the freezing tests exposed the tissues to considerably more stress than the slower cooling rates used in most other investigations. It also shows that it is necessary to use much lower test temperatures when slow temperature-change techniques are used to determine the degree of hardiness in the material, than with the technique used in the present work.

Relation between visible damage and relative conductivity

In order to elucidate the correlation between the values obtained through conductivity measurements and the visible injuries appearing after storage of the shoots for some time, the following experiment was performed. Shoots of two year old plants were freeze-tested on six different occasions between January and May 1969. Some of the shoots from each treatment were used for conductivity measurements as described in *Methods*, while the rest of the shoot sample was kept at room temperature in front of a window with the bases in water. Control shoots were maintained uninjured under these conditions while the frozen shoots showed varying degrees of injury.

The samples were numbered and the symptoms of injury were evaluated after four or five weeks without knowledge of the treatment given to the individual sample. The following symptoms were estimated and given values from 1—4, where 1 is unchanged and 4 denotes maximal injury or change from the normal:

- Discoloration in the interior of the buds.
- Ease of abscission of the buds. (Buds on damaged shoots loosened easily).
- Discoloration of the needles.
- Ease of abscission of the needles.

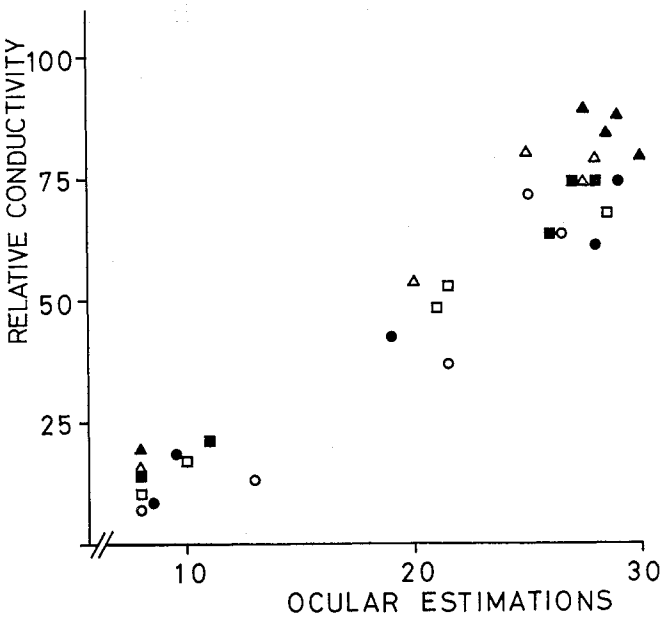

Fig. 5. Comparison of Relative Conductivity and injury estimates by ocular inspection, carried out on the same samples. Average values of Relative Conductivity for buds, needles and stems (ordinate) and sum of ocular ratings for various visible symptoms (see the text; abscissa). Freezing treatments were made at +4°C (control), —12°C, —22°C, —32°C, and —44°C. Symbols: ○ 6 Jan.; ● 3 Feb.; □ 24 Feb.; ■ 12 April; △ 5 May; ▲ 26 May.

– Dryness of the needles.
– Discoloration of the cambial zone of the stems.
– Softness of the cambial zone.
– Discoloration in the wood (only the values 1—2).

The sum of the values will be 8 for undamaged shoots and 30 for completely killed shoots.

The values obtained in the conductivity measurements and by ocular estimation of the injuries are plotted in Figure 5. A close correspondence was obtained between the results of the two methods. The correlation coefficient calculated for the numerical values obtained with the two methods is 0.95. Other comparisons carried out with other pine materials gave similar results. Potted intact plants frozen in the same way, but with the roots protected by an insulating covering, showed the same sensitivity in the freezing tests as the excised shoots used in this investigation.

Variation in hardiness of pine during the winter season

The changes in frost hardiness were followed in Scots pine during the winters 1967—68 and 1968—69. After freezing tests on the whole shoots or twigs, conductivity was determined separately for buds, stems and needles. Some of the results obtained are shown in Figures 6—8.

Figure 6 shows the RC values obtained for needles from three 6—8 m high, about 20 years old pines growing solitarily at the experimental station at Bogesund, 20 km north-east of Stockholm. The freezing treatments were carried out with the previous year's growth from twigs 2—4 m above ground level. Each test comprised four randomly selected twigs from the same tree. The routine for the conductivity determinations was not completely worked out when the first tests were made in October. This is the reason for some irregularities in the values obtained on the first two occasions. In most of the tests the values for the trees agree closely. The change in hardiness during the winter is that reported for related species in several earlier investigations (Ulmer, 1937; Pisek, 1950; Parker, 1955). From 23 October until 16 April the needles were resistant to freezing at —12°C. From 27 November until 25 March the treatment in the —22°C deep-freeze caused none or insignificant injury. A decrease in the injury caused by the —44°C treatment was evident in December and February. This decrease indicates increased hardiness during mid-winter. As is indicated in the temperature curves, this increase coincides with the coldest period.

Figure 7 represents similar plant material growing in the canopy in the vicinity of the trees used for the determinations of Figure 6. In this case, one twig from each of ten trees was used in the freezing treatments. Conductivity tests were carried out on pooled samples of buds, stem sections and needle sections from the ten shoots. Figure 7 provides a comparison of the response of buds, stems and needles to the freezing tests. While the stems and needles respond in a similar manner, the buds seem to be more hardy and to deharden more slowly in spring. Since, with the method used, the rate of cooling is different for different organs, no conclusive statement can be made regarding real differences in frost hardiness between different organs. It may be pointed out, however, that the cooling rate in nature is different in different organs. While the temperature fall in the needles may be very rapid under certain circumstances, a slower cooling rate may be expected, especially in the buds, where the bud scales may provide some insulation. Twigs obtained from pines growing in northern Sweden (Gällivare) in winter showed greater frost hardiness. Only small injuries were

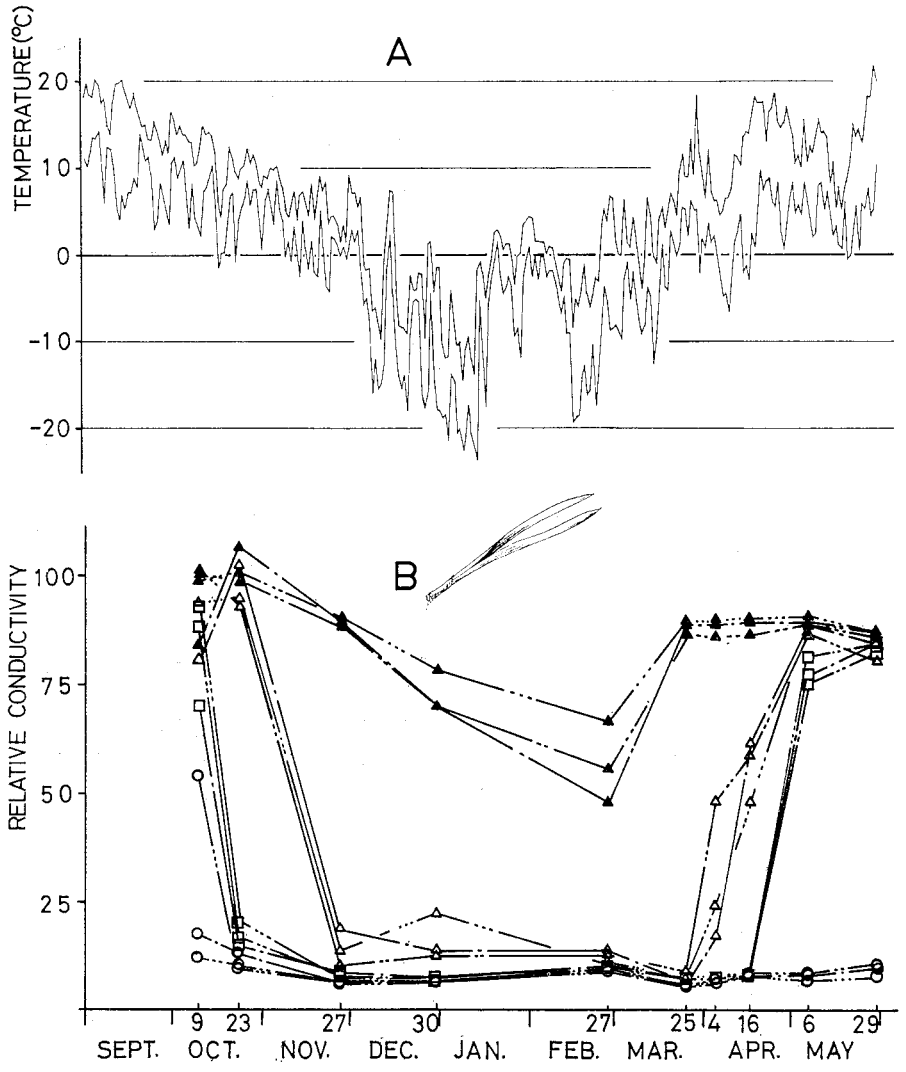

Fig. 6. Variation during the winter 1967—68 in RC values for needles from three 20 year old pines growing at Bogesund near Stockholm (B). The values for each tree shown separately. The daily minimum and maximum temperature as recorded at the Meteorological station at Bromma are shown (A). Freezing treatments: ○ Control; ▯—12°C; △ —22°C; ▮—32°C; ▲ —44°C.

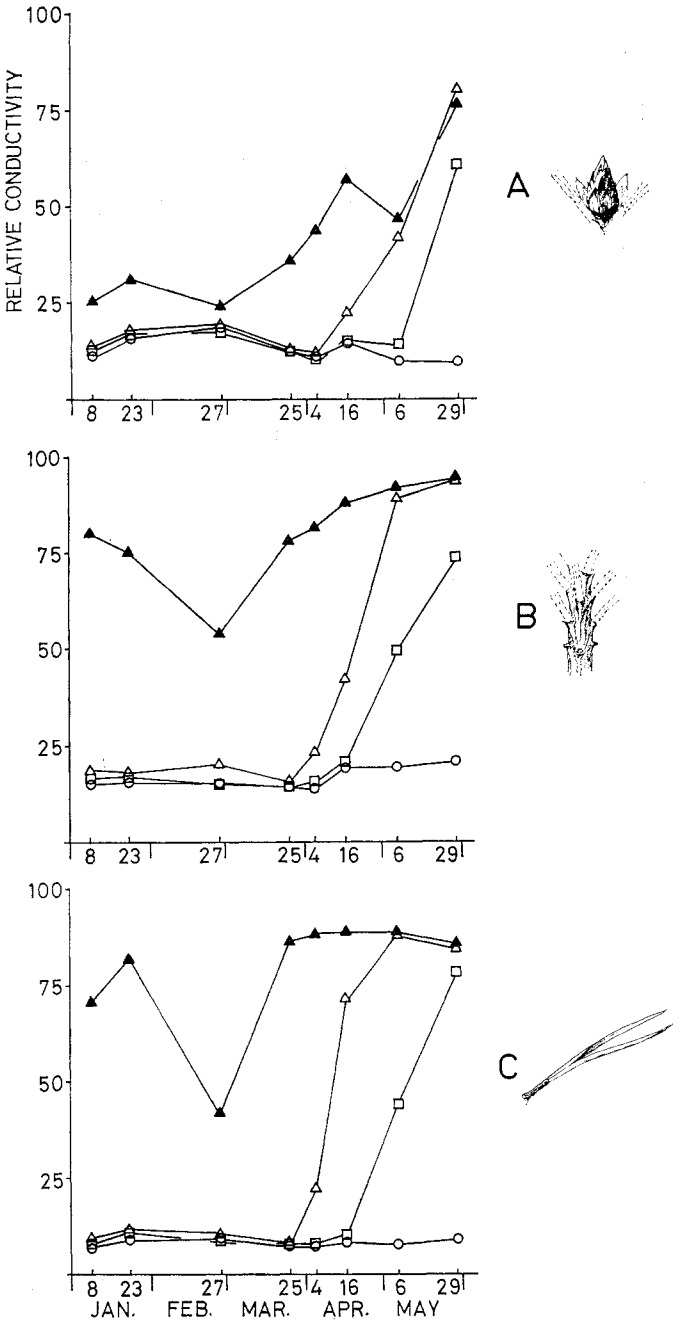

Fig. 7. Variation from January to May 1968 in RC values for buds (A), stems (B) and needles (C) of twigs from ten pine trees in a canopy at Bogesund. Symbols as in Fig. 6.

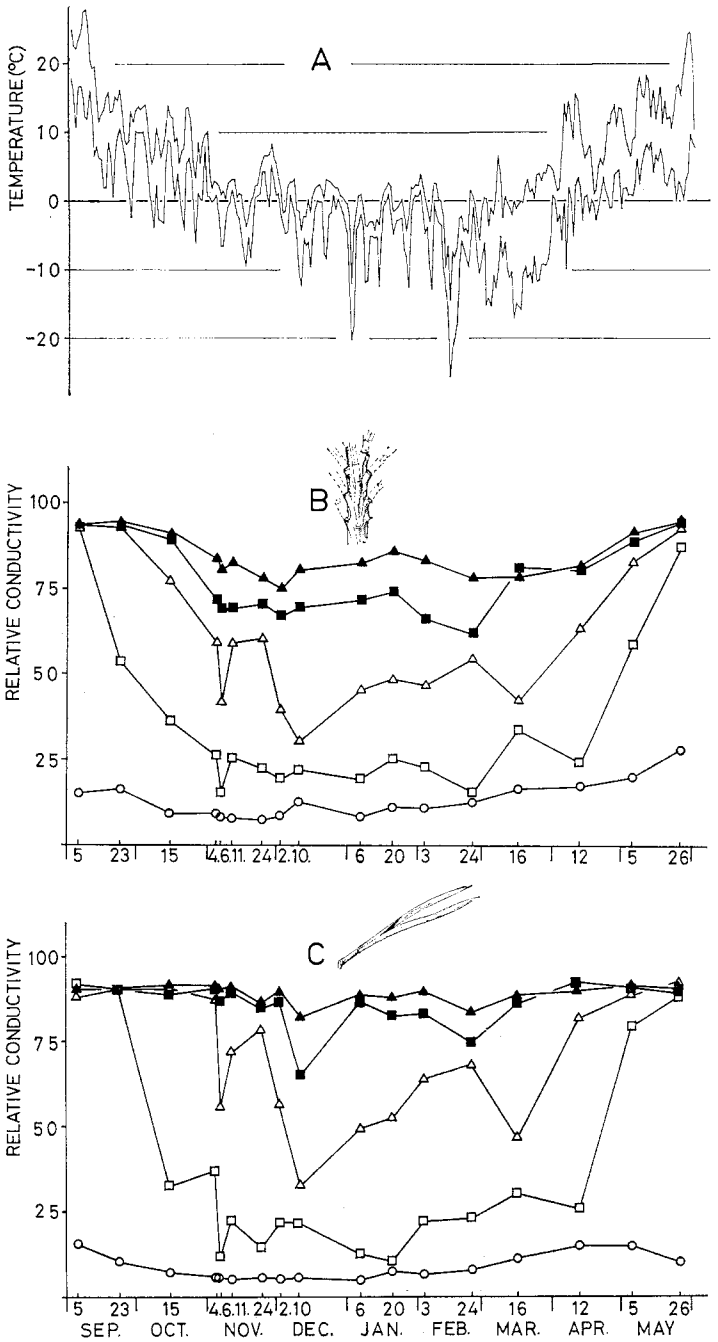

Fig. 8. Variation in RC values in stems and needles of two year old pine seedlings during the winter 1968—69.
A. Daily minimum and maximum temperatures.
B. RC values for stems.
C. RC values for needles.
Symbols as in Fig. 6.

Table 3. Rainfall and temperatures in Stockholm as recorded at the meterological station at SMHI, Kungsholmen, from 28 Oct. to 11 Nov. 1968. Samples of two year old seedlings brought indoors for hardiness determinations at 16.00 h on the dates marked with X (see also Fig. 8)

	October				November										
Date	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11
Rainfall, mm	6.6	8.9	1.4	29.5	4.2	4.5	1.5	0.6		0.0	1.0	0.4	4.1	0.9	0.8
Sun, hours			3.4						7.7	7.5	1.3				
Temperature															
max	8.4	9.3	9.7	4.4	2.8	2.8	2.7	2.4	—0.2	0.5	0.1	2.4	2.9	3.3	3.3
min	—0.4	7.6	3.2	0.8	—0.4	0.1	1.0	—2.7	—5.8	—3.6	—2.0	—0.7	—0.2	0.1	0.2
Hardiness determination								×		×					×

caused to this material by the —44 °C treatment (unpublished experiments by A. ARONSSON).

Figure 8 shows the variation in RC valus 1968—69 obtained with two year old (2/0) seedlings grown in a plastic green-house during the summers 1967 and 1968 at Ågrena experimental nursery. These plants were supplied by the Department of Reforestation. Seeds were obtained from Vimmerby in Småland, 90 m altitude. The seedlings were transplanted into containers in August 1968 and moved to the Royal College of Forestry, Stockholm. They were stored out of doors during winter 1968—69, snow being removed from them when necessary.

The hardiness of these plants was markedly lower than that of the older pines investigated. A short-term variation in hardiness, influenced by weather changes, is indicated by the values obtained. This may be exemplified by the sudden increase in hardiness from 4 to 6 November, followed by decreased hardiness on the 11 November. The increase in hardiness was induced by two sunny days with night temperatures down to —5,8 °C (Table 3). Some dehardening occurred on the following cloudy days with light rain and temperatures slightly above 0 °C. In this material probably both rapid hardening and dehardening occurs in response to change in the weather throughout the winter. Tests at closer intervals than those generally used in the present investigation are, however, necessary to follow short term variations in hardiness. One year old seedlings were also investigated. They showed somewhat less hardiness than the two year seedlings, especially as regards the needles.

Discussion

The freezing test used in the present investigation is at variance with the established practice of using a slow and constant rate of cooling (LEVITT, 1956). There are several reasons for the selection of the technique used.

The first reason is that the conventional tests consider only one aspect of frost hardiness. As was discussed by LEVITT (1956) and more recently by MAZUR (1969), intracellular freezing does not occur at the low cooling rates used in most freezing tests. These tests for this reason only measure the ability of the cells to endure dehydration. It is by no means certain that this is the property of most significance for frost hardiness in nature. The most serious frost damage is probably caused either by sudden temperature falls which occur periodically throughout the cold season (PARKER, 1955) or by changes between day and night temperatures occurring in clear and sunny weather. Solar radiation may raise the temperature of leaves and bark several degrees above the air temperature. At sunset the rate of temperature fall may be considerable. WHITE & WEISER (1964) reported rates of temperature fall of the order of 9°C (17°F) per minute in the foliage of American arborvitae at sunset. Injuries caused by such rapid temperature decreases are most probably due to intracellular ice formation. Although the temperature fluctuations are generally less pronounced (SAKAI, 1966), resistance to intracellular freezing is certainly an important component of frost hardiness in nature. There is thus a strong reason for including hardiness to rapid temperature changes in a freezing test, as was done in the method described in the present paper.

A second reason, pertinent to very hardy woody plants, is that a freezing test with a low rate of cooling will not work because the tissue will endure even very low temperatures. TUMANOV & KRASAVTSEV (1959), SAKAI (1960) and PARKER (1962) showed that not even the temperatures of liquid nitrogen (—196°C) or helium (—269°C) will kill very hardy plant tissues. It is possible, however, to obtain relative values for frost hardiness for such plant tissues by varying the course of temperature lowering. SAKAI (1965) used the varying ability of tissues pre-cooled to temperatures from —15°C to —30°C to endure transfer to liquid nitrogen as a measure of their frost hardiness. In the freezing procedure used in the present work the problem is solved by varying not only the minimum temperature but also the rate of cooling.

Another objection to the use of slow cooling rates would be that an appreciable hardening may occur during the period of temperature decrease.

TUMANOV & KRASAVTSEV (1959) showed that a considerable additional hardening of woody plants hardened under natural conditions was obtained by slow stepwise cooling. This means that the ability of the plant to harden rapidly at temperatures below freezing-point will affect the results in conventional freezing tests. Such rapid hardening certainly occurs in nature, and it is of course of importance to the ability of the plant to withstand the winter. It is obvious, however, that the ability to harden rapidly at temperatures below freezing is a property which should be measured separately from the actual hardiness of a plant.

The method of cooling the plant tissues in deep-freezes maintained at predetermined temperatures represents a technically simple way of avoiding the above-mentioned shortcomings of the conventional methods. From a theoretical point of view, determination of the response to different rates of temperature lowering is desirable (MAZUR, 1969), but this would demand much more elaborate equipment. An important consideration in working out the experimental technique has been that the method should be suited to large-scale tests with the apparatus installed in a caravan. It is recognised that the freezing method is open to the criticism that cooling rate is dependent on the thickness and the heat conductance of the plant organs. Cooling will probably be more rapid in the needles than in the stems and buds. For this reason it is not possible to compare exactly by this method different kinds of tissue from the same plant. It is suggested that the method is most suitable for hardy plant materials.

Injuries caused to the tissue during freezing may be satisfactorily evaluated by several techniques. Observational methods are the most direct and are often facile. While it is often easy to classify the plant samples as dead or living by this method, it is more difficult to rank objectively injuries to material which is only partly damaged. For stem parts of woody plants with buds which will sprout rapidly if the tissues are not damaged, the method is convenient (SAKAI, 1965). For dormant shoots it may be found less satisfactory. In shoot tissues of conifer species, visible symptoms will develop only slowly and the environmental conditions during storage may affect the degree of injury. Hence, if the test is to be reproducible, the plant material must be stored after freezing and thawing under controlled conditions of temperature, light and air humidity. The fact that the degree of injury must be subjectively assessed may cause unpredictable errors in comparisons of the hardiness on different occasions. Plasmolysis tests and staining methods are rather time-consuming. They are most useful for exactly localising the injury in the tissue. The *determination of conductivity*, however, is a rapid and simple method which will give objective values for the degree of injury. In this investigation the method has been found satis-

factory. The procedure, however, must be strictly standardised, to make the values from different tests comparable.

Combined with the conductivity method for evaluation of the degree of injury, the freezing test has given reproducible values for frost hardiness in Scots pine. The changes in hardiness during the winter season show good agreement with those obtained by other freezing tests in earlier investigations. It is evident that rather small differences in hardiness may be accurately determined. It is believed that the method will be of value in studying how various factors influence the degree of hardiness in forests trees during the cold season.

ACKNOWLEDGEMENTS

Investigations on winter hardiness of conifers form an important part of the research programme of the Royal College of Forestry. It is not possible here to mention all persons who have contributed in various ways to the results reported in this paper. We are particularly grateful to professor C. O. Tamm for initiation of the investigation and for discussion and support during the work. Experimental material was provided by the Department of Reforestation (head professor G. Sirén) and a linguistic revision of the manuscript was made by Mr J. Flower-Ellis.

Summary

Methodological problems in the investigation of frost hardiness of plants are treated. The most important methods used in this field are surveyed. A relatively simple procedure for determining the degree of frost hardiness in hardy woody plant tissues has been worked out, using seedlings and trees of *Pinus silvestris* (L.) as experimental plants.

Before the freezing test, shoots or twigs of the plants are subjected to a temperature of +4°C in a refrigerator. They are then placed in deep-freezes maintained at predetermined temperatures (—12°C, —22°C, —32°C and — 44°C) for six hours. Temperature lowering in the plant tissues is rapid under the conditions used, the temperature of the deep-freeze being reached in slightly over ten minutes. The rate of cooling depends on the final temperature. The stress on the plant tissue is thus greater at lower temperatures, not only because of the lower final temperature but also because of the more rapid rate of cooling. It is suggested that this reproduces conditions in nature, where frost damage may appear either in consequence of dehydration of the protoplasm due to ice formation in the intercellular spaces (which will increase with decreasing temperature) or in consequence of ice formation in the protoplasm (which occurs on rapid cooling). The freezing test described differs from conventional tests in respect of the rapid rate of cooling (up to 6°C per minute in 4 mm thick stems). The following arguments are adduced to support the use of the method introduced here:

1. Not only the ability of the cells to endure dehydration, but also their resistance to intracellular ice formation, will influence the result of the test.
2. It is also possible to test very hardy plant tissues without using extremely low temperatures.
3. Actual frost hardiness is measured, because no hardening will have time to occur during the cooling period.
4. The method is technically simple. It is suitable for large series and may be used in field laboratories.
5. Rapid cooling also occurs in nature, when sun-exposed branches are shaded at low air temperatures.

The injuries caused to the tissue in the freezing test are evaluated by the determination of electrical conductivity. Sections of needles and stems or whole buds are placed in distilled water. The conductivity of the solution will depend on the degree of injury of the tissues, because electrolytes will diffuse from killed or injured cells to a greater extent than from normal cells. A reference value for completely dead tissue is obtained by a second determination of conductivity after killing of the tissue by boiling. The conductivity of freeze-tested tissue, expressed on a percentage basis (Relative Conductivity, RC) is a convenient and reliable measure of the degree of injury. Good agreement was obtained between RC determined shortly after the freezing test and the degree of visible injury appearing on the shoot after four or five weeks.

The methods described have been used for determining the changes in frost hardiness from October to May under the climatic conditions of the Stockholm region. Relatively small changes in frost hardiness induced by short-term variations in the weather may also be determined by the procedure.

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Sammanfattning

Frosthärdighet hos tall (*Pinus silvestris L.*)

I. Metoder för testning av härdigt plantmaterial och för bestämning av skador med ledningsförmågemätningar

Uppsatsen behandlar främst metodiska problem vid undersökningar av frosthärdigheten hos växter. En översikt har gjorts över de viktigaste inom detta område använda metoderna. Nödvändiga moment i sådana undersökningar är behandling av växtmaterialet med låg temperatur (frystest) samt bestämning av resultatet av denna behandling. Ett relativt enkelt förfarande, som lämpar sig för bestämning av graden av frosthärdighet särskilt hos härdigt material av vedväxter har utprövats med tallplantor av olika ålder som försöksväxt.

Frystestet utföres på så sätt att skott eller kvistar av plantorna först får antaga temperaturen +4°C i ett kylskåp. De placeras sedan 6 timmar i frysboxar, som redan från början har fått antaga olika temperaturer (—12°, —22°C, —32°C och —44°C). Temperatursänkningen hos växtmaterialet sker under de använda betingelserna snabbt. Redan efter ca 10 minuter har kvistarnas temperatur sänkts till i närheten av frysboxens temperatur (Figur 2). Ju lägre temperaturen i frysboxen är ju snabbare blir temperatursänkningen per tidsenhet. Den påfrestning växtmaterialet utsättes för blir alltså större vid lägre temperaturer, inte bara därför att sluttemperaturen är lägre utan också genom att nedkylningshastigheten är större. Metoden avviker från konventionella frystest genom den snabba temperatursänkningen (upp till 6°C per minut i 4 mm grova stammar). Vanligen kyles materialet i dylika test med en hastighet av 1—5°C per timme.

Det är känt, att köldskador — även i naturen — uppstår antingen som följd av dehydratisering (uttorkning) av protoplasman genom isbildning i intercellularerna (blir kraftigare ju lägre temperaturen är) eller som följd av iskristallbildning i själva protoplasman (beror på snabb temperatursänkning). Följande skäl kan anföras för användning av den här introducerade metoden:

a. Inte bara cellernas förmåga att uthärda dehydratisering utan även deras resistens mot intracellulär isbildning ger utslag i testet.
b. Det är möjligt att testa även mycket härdigt material utan att behöva tillgripa extremt låga temperaturer.
c. Testet visar den aktuella härdighetsgraden eftersom ingen härdning hinner ske under själva nedfrysningen.
d. Metoden är tekniskt enkel och arbetsbesparande. Den lämpar sig därför för stora serier och kan användas även i fältlaboratorier.
e. Snabba nedfrysningsförlopp förekommer även i naturen, t. ex. när vid låga lufttemperaturer solbelysta barr kommer i skuggan.

Skador uppkomna vid frystestet bestämmes genom *ledningsförmågemätningar*. Sektioner av barr och stammar respektive hela knoppar placeras i destillerat vatten. Ju större skadorna på vävnaderna är ju högre blir den

elektriska ledningsförmågan hos lösningen, eftersom elektrolyterna diffunderar ut från döda celler mycket lättare än från levande. Ett referensvärde för helt dött material erhålles genom att en ny bestämning av ledningsförmågan utföres, sedan vävnaderna dödats genom kokning. Det värde på ledningsförmågan, som erhållits efter enbart frystestet, uttrycks i procent av ledningsförmågan för samma prov med vävnaderna helt döda. På så sätt erhålles ett bekvämt och tillförlitligt mått på graden av skada, här kallat relativa ledningsförmågan (RC). God överensstämmelse erhölls mellan relativ ledningsförmåga bestämd i anslutning till frystestet och synliga skador på skott, som fått stå 4—5 veckor.

De beskrivna metoderna har använts för bestämning av förändringar av frosthärdigheten från oktober till maj för några olika tallmaterial under de klimatiska förhållanden, som råder i Stockholms-trakten. Resultaten framgår av figurerna 6—8. Även relativt små förändringar i härdighetsgraden inducerade av korttidsvariationer i klimatet kan bestämmas med dessa metoder.