New Phytol. (1999), **144**, 73–83

Effects of excess nitrogen on frost hardiness and freezing injury of above-ground tissue in young oaks (*Quercus petraea* and *Q. robur*)

F. M. THOMAS* AND U. AHLERS

Universität Göttingen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Abteilung Ökologie und Ökosystemforschung, Untere Karspüle 2, D-37073 Göttingen, Germany

Received 22 December 1998; accepted 2 June 1999

SUMMARY

The effects of excess nitrogen (N) on the frost hardiness and freezing injury of bark and buds were tested in 2-yr-old sessile oaks (*Quercus petraea*) and pedunculate oaks (*Q. robur*) that had been grown outdoors in sand culture with normal or luxurious N supply during the growing season. Some trees from both N treatments were subjected to drought stress in summer, whereas others were adequately watered. Between January and March, whole plants were exposed to artificial freezing treatments at −25°C of different durations (10 d, 21 d, or two periods of 4 d interrupted by an 8 d frost-free period, referred to as a freeze–thaw cycle). The frost hardiness and freezing injury of the bark were determined by two different versions of the electrolyte leakage method (calculation of an index of injury, I_{-25}, from the relative conductivity after a fixed time; and measurement of the electrolyte leakage rate). In addition, the vitality of bark and buds was assessed visually with the 2,3,5-triphenyltetrazolium chloride (TTC) method. In comparison with the control, the oaks that had been luxuriously supplied with N exhibited distinctly higher N concentrations and lower C : N ratios in their leaves and bark, higher relative shoot increments, delayed leaf discoloration in autumn, and earlier budbreak in spring. Oaks kept outdoors during the winter were frost-hardy from December until the end of February without showing differences between species or N treatments. In comparison with the pedunculate oak, the sessile oak was more susceptible to freezing injury induced by prolonged artificial freezing or by the freeze–thaw cycle. Increased freezing injury in trees with high N supply was detected only by the TTC test of the bark of sessile oaks subjected to the freeze–thaw cycle. In all oaks, except for the pedunculate oaks grown with normal N, the freeze–thaw cycle resulted in significantly lower frost hardiness, as indicated by increased I_{-25} values. This treatment also led to increased freezing damage of bark and buds, as revealed by the TTC test. In drought-stressed oaks, the electrolyte leakage rate was significantly elevated after the freeze–thaw cycle. It is concluded that, in the sessile and pedunculate oaks, the water supply before frost stress and the course of the temperature in winter have a greater effect on frost hardiness and freezing damage than excess N.

Key words: bark, drought, frost hardiness, nitrogen, *Quercus* (oak).

INTRODUCTION

In temperate and boreal climates, the ability of perennial phanerophytes to tolerate winter frost is a decisive feature for their survival and propagation. The periodic development of frost hardiness is an acclimation process, which is induced by decreases in day length and low temperatures. In winter, the extent of frost hardiness is governed largely by the current temperature (Sakai & Larcher, 1987). However, it also depends on the nutritional status: both nutrient deficiency (especially potassium; Levitt, 1980) as well as an excess of nutrients (N; Levitt, 1980; Larcher, 1985) can diminish the frost hardiness. A decrease in frost hardiness owing to a supply of excess N has been found in young and adult Scots pines and in adult Norway spruce (Aronsson, 1980; Soikkeli & Kärenlampi, 1984). Prolonged drought, which decreases the carbon gain, can also result in decreased frost resistance (Sakai & Larcher, 1987).

Deep frost in three consecutive winters in the mid-1980s was proposed as a synchronizing factor

*Author for correspondence (tel +49 551 395724; fax +49 551 395701; e-mail fthomas@gwdg.de).

for the occurrence of damage to the oak in large parts of Europe (Schlag, 1994). In nearly all stands of pedunculate oak (*Quercus robur*) and sessile oak (*Q. petraea*) investigated in northern Germany, up to 20 % of the severely damaged trees exhibited primary bark necroses, which were caused by minimum temperatures down to −26°C in January and February (Hartmann & Blank, 1993). Because of anthropogenic deposition of excess N, most oak stands studied in northwestern Germany show elevated foliar N concentrations compared with published data (Thomas & Büttner, 1993; Thomas & Kiehne, 1995). During frost periods in January or February, bark tissue sampled from mature oaks exhibited decreased frost hardiness when the C:N ratios were significantly decreased; a significant correlation was found between frost damage to the bark and its C:N ratio (Thomas & Blank, 1996). In that study, however, the effects of site factors could not be excluded. An investigation was therefore initiated on the effect of excess N on the frost hardiness of above-ground tissue of young oak trees under controlled conditions. Because summer drought is also supposed to be one of the primary causal factors of damage to oaks (Schlag, 1994), a proportion of the trees was subjected to drought stress before the freezing treatment. To test the reaction of the oaks to sequential freezing and thawing, some of the plants were subjected to a freeze–thaw cycle during the freezing treatment.

The frost hardiness and the extent of the freezing damage to the bark tissue were quantified by two different versions of the electrolyte leakage method. To assess the frost hardiness of plants, Flint *et al.* (1967) suggested calculating an index of injury from the relative electric conductivities of solutions in which the samples had been incubated. This method has been widely adopted to test the frost hardiness of various parts of trees such as stem sections (van den Driessche, 1976), twigs (Alexander *et al.*, 1984) and needles (Burr *et al.*, 1990). More recently, Murray *et al.* (1989) developed a method based on the rate of electrolyte leakage, which has been used successfully to quantify the freezing injury to woody shoots in, for example, *Calluna vulgaris* (Caporn *et al.*, 1994), *Quercus petraea* (Deans *et al.*, 1995) and *Picea abies* (Sheppard *et al.*, 1995). In our investigation we compared both methods with a visual assessment of the freezing damage, which was performed with bark and buds after incubation with a solution containing 2,3,5-triphenyltetrazolium chloride (TTC).

MATERIALS AND METHODS

Plant cultivation

In the first week of May, 100 2-yr-old pedunculate oaks (*Quercus robur* L.) and 100 2-yr-old sessile oaks (*Q. petraea* (Matt.) Liebl.) obtained from a tree nursery (with a provenance in the lowlands of northern Germany) were uplifted and put into sand culture. Each plant was potted into a polyethylene vessel as described by Kick & Grosse-Brauckmann (see Baumeister & Ernst, 1978), which was filled with 8 l of a mixture of coarse (40 %, w/w) and medium (53 %, w/w) sand. The pots were placed outside. In the event of precipitation, the plants were sheltered by an automatically controlled roof.

Starting in the third week of May, the plants were fertilized every 2 wk with 500 ml of nutrient solution per vessel. Fifty plants per species were supplied with a solution containing 1.5 mM N in the form of NH_4NO_3. This treatment is referred to as 'normal N'. The other fifty plants were grown with 'luxurious N', which was performed with a nutrient solution of 12 mM N (given as NH_4NO_3). The concentrations of the other nutrients were the same in both types of solution (in mM: K 6.0, Mg 2.1, Ca 0.9, P 0.5, S 7.3; in μM: Fe 135, B 13.5, Mn 7.5, Zn 6.0, Mo 3.6; pH 4.8). As a result of analyses of the foliar N concentrations, which were conducted at the end of June, the N supply was decreased in the 'normal N' treatment (once by 25 % and twice by 50 %), and increased in the 'luxurious N' treatment (four times by 50 %) on the subsequent fertilization dates to adjust the plants' N contents to the desired levels. When summed over the entire growing season, the total N supply was 2.12 g m^{-2} in the 'normal N' treatment and 24.3 g m^{-2} in the 'luxurious N' treatment.

Immediately after fertilization, the plants were watered with demineralized water to prevent salt injuries. The water content of the substrate was maintained at 70 % of its water-holding capacity by regular watering. From the beginning of July to the end of August, four oaks per species and N treatment were kept at only 30–40 % of the substrate's water-holding capacity to simulate drought stress. In early September, several leaves were harvested from each plant for the measurement of nutrient concentrations. After the leaves had been dried at 105°C and pulverized, their C and N concentrations were determined with a C–N analyser (NA 1500; Carlo Erba, Rodano/Milan, Italy). The K and Mg concentrations were measured by atomic absorption spectrometry after acid digestion of the dried and pulverized leaf material. At the end of the growing season, the shoot lengths of 40–50 oaks per species and treatment were measured, and related to their shoot lengths at the start of the experiment to calculate the relative shoot increment.

During the winter, the oaks remained outside. The humidity of the substrate was regularly controlled to prevent desiccation. At a height of 50 cm above the ground, the air temperature was recorded with a temperature sensor (LI 1000–15; LI-COR, Lincoln, NE, USA), which was connected to a data logger (LI-1000; LI-COR).

CAMBRIDGE UNIVERSITY PRESS

Freezing treatments

The freezing treatments were performed in a programmable freezing cabinet and were started between the beginning of January and the beginning of March. They were conducted in the dark to prevent any disturbance of the freezing programme by heating through lamps. The plants were frozen in their pots. To prevent freezing damage to the roots, dry leaf litter was put into the spaces between the pots and on the surface of the substrate. In all treatments, the starting temperature within the cabinet was +5°C. The plants were cooled with a cooling rate of 1.25°C h^{-1} down to a final temperature of −25°C. Temperatures of approx. −25°C for several days have been suspected to causing primary bark necroses in oaks growing in different regions of northern Germany (Hartmann & Blank, 1993). At −25°C, the rh within the freezing cabinet was approx. 45 %. At the end of the respective treatments, the temperature rose at a rate of 2.5°C h^{-1} up to the initial temperature of +5°C. The duration of the treatments was 10 d (treatment A), 21 d (treatment B) or 20 d (treatment C). In treatments A and B, the final temperature was kept constant at −25°C for the entire period. In treatment C, the oaks were subjected to a freeze–thaw cycle. Starting on day 5 of the treatment, the temperature was elevated at a rate of 0.73°C h^{-1}, resulting in a temperature of +10°C on day 6. This temperature was maintained for the subsequent 8 d. In northern Germany, relatively warm periods with daily minimum air temperatures of up to +10°C are not unusual during winter, and can interrupt periods of severe winter frost (cf. Thomas & Blank, 1996). On day 15 the temperature was lowered again at a rate of 0.73°C h^{-1}, ending at −25°C on day 16. This temperature was then kept constant until day 20, when the treatment was terminated by increasing the temperature to the final value as already stated.

For all treatments, eight trees per species and N treatment were used. In treatment C, half of the plants had been adequately watered (referred to as C_W), and the other half (denoted C_D) had experienced drought stress in the preceding summer (already described). All 16 plants of treatment C were subjected to this treatment at the same time.

Sample preparation for assessment of frost hardiness and freezing damage

At the end of the freezing treatments, the shoots of the plants were harvested, wrapped in moist tissue and put into polyethylene bags to prevent desiccation, and transferred to the laboratory in a cooling box. There, the stems were rinsed with demineralized water and blotted dry. From the periderm to the cambium of each plant, strips approx. 30 mm long, 3–4 mm wide and 1–2 mm thick were cut out of the bark. Three strips each were placed into one capped vial. In addition, on five dates between the beginning of December and the end of April, three or four plants per species and N treatment that had remained outside throughout the entire investigation were harvested. In these plants, the frost hardiness of the bark tissue during the winter was recorded by artificial freezing at −25°C (see later). The preparation of bark samples was performed in the same manner as for the oaks subjected to the freezing treatments. The vials were kept at +5°C until further processing.

Electrolyte leakage

For the assessment of the frost hardiness of the plants that had been frozen in the cabinets and those kept outside, four vials per plant were prepared as described above, then treated in the same manner as the bark samples from adult oaks in a previous investigation (Thomas & Blank, 1996): on the day after plant harvest and sample preparation, two vials per plant were frozen in a cryostat (Fryka FT 10–44; National Lab., Mölln, Germany) from +5 to −25°C at a cooling rate of 5°C h^{-1} (as described by Kolb *et al.*, 1985). At 30 min after the final temperature was reached, the samples were removed from the cryostat and left to thaw overnight in a refrigerator at +5°C. The remaining two vials per plant were kept as control samples in the refrigerator during the freezing procedure. The electrolyte leakage caused by the freezing treatment in the cryostat was determined in accordance with Ritchie (1991): after thawing, the frozen and the control samples were infiltrated under vacuum with 6 ml of 3 % (v/v) propanol in distilled water and incubated for 24 h at 25°C. After incubation, the conductivity of the medium was measured (conductivity sensor LTA 1, conductometer LF 2000/C; WTW, Weilheim, Germany) and the tissue was killed by autoclaving at 120°C for 15 min. The incubation and the conductivity measurement were then repeated. The index of injury, I_{-25}, caused by freezing at −25°C was calculated from the ratios of the conductivity values before and after autoclaving obtained from treatment and control samples (after Flint *et al.*, 1967). The maximum range of this index was 0 % (no freezing damage) to 100 % (tissue completely killed by freezing). In previous investigations on mature oaks it was shown that the artificial freezing of bark samples at −25°C with the above procedure was not sufficient to completely kill the tissue sampled from December to April, but yielded I_{-25} values between approx. 5 % and 60 %, which also exhibited a good correlation with the temperature course during winter (Thomas & Blank, 1996).

In addition to the determination of the I_{-25} values, the damage to all of the plants that had been frozen in the cabinets (see previously) was assessed by

CAMBRIDGE UNIVERSITY PRESS

measuring the rate of electrolyte leakage by the method of Murray *et al.* (1989). This was done because some authors (i.e. Deans *et al.*, 1995) found that the determination of the rate of electrolyte leakage yields results that are more consistent than those obtained from the measurement of the relative conductivity after only one fixed time. Two vials per plant, which had been prepared as described above, were used for the test. After the addition of 15 ml of distilled water and thorough mixing, the conductivity of the solution was measured (see previously). Further measurements of the conductivity were performed after 3, 6, 9, 21, 24, 48, 72 and 96 h. Between measurements, the samples were kept at +5°C to minimize microbial activity. After the measurement at 96 h, the samples were killed by autoclaving (15 min at 120°C); 4 h later, the final measurement of conductivity was performed. Preliminary investigations had shown that the duration of the 4 h incubation period before the final measurement was sufficient to yield maximum conductivity values. Relative conductivities (RC; as percentages) were calculated from the ratios of the conductivity at the different times of incubation and the conductivity after autoclaving. Even after only 9 h of incubation, the RC was close to its maximum, probably because of the high surface : volume ratios of the bark samples. Therefore, only the data measured during that time were considered for the following computation. The RC values were plotted against the logarithms of the values of the time of measurement (in hours). The slope b of the regression ($RC = a + b \ln t$) was computed by linear curve fitting (Multigraf 3.02; Midas, Frankfurt/Main, Germany), and was taken as a measure of the electrolyte leakage rate. With our data, this empirical regression yielded higher correlation coefficients than the theoretically derived equation used by Murray *et al.* (1989). Increased values of b would indicate a higher electrolyte leakage rate and thus an increased freezing damage (Murray *et al.*, 1989).

For determination of the C and N concentrations, bark samples from the oaks subjected to the freezing treatment as well as from oaks kept outside were prepared as described above, frozen in liquid N_2, and kept frozen until further processing. After the bark samples had been dried at 105°C and pulverized, their C and N concentrations were measured as already described. One combined sample per freezing treatment, and per date of harvest of plants kept outside, was made.

Reduction of TTC

In addition to the electrolyte leakage methods, the frost damage to the oaks was assessed visually by means of the histochemical reduction of TTC to 2,3,5-triphenyltetrazolium formazan, as described by Larcher (1969). Two or three bark strips and buds were taken from plants, which were kept outside, on the starting dates of the different freezing treatments, and from plants after the respective freezing treatments. The buds were cut longitudinally. The samples were vacuum-infiltrated for 30 min with a solution of 0.5 % TTC in demineralized water and incubated for 24 h at 30°C. The samples were then rinsed thoroughly with water and their coloration was assessed with a dissecting microscope. Bright red, red or purple samples were assessed as vital, samples with a weakly to patchy red coloration as less vital, and samples with a weakly or patchy red to brownish colour as subvital. Buds were classified as subvital if the insertion in the stem was still red but the leaf primordium was brown. Completely brown samples were assessed as dead.

Statistics

In the presentation of the results, means and standard errors are given. The differences in the relative shoot growth increments, and in the foliar concentrations and ratios of nutrients, between the N treatments were tested with the t-test, or with the U-test when there were only four replicates (drought-stressed plants). For the frost-hardiness assessment of plants kept outside, fewer than four plants were available on some dates. The non-parametric H-test after Kruskal & Wallis (cf. Sachs, 1984) was therefore used to test differences in the I_{-25} values between the single species and N treatments on a given date.

In most cases the I_{-25} and b values of the various frost treatments were normally distributed. This was tested by using the UNIVARIATE procedure (SAS 6.04; SAS Institute, Cary, NC, USA) and the distribution of the W values (significance level $P < 0.1$ (Shapiro & Wilk, 1965)). Three approaches were made to the assessment of the effects of species, cultivation and freezing treatment on frost hardiness and freezing damage: (1) differences between species and N supply in I_{-25} and b within the single freezing treatments were tested with one-way ANOVA (SAS 6.04); (2) differences between the freezing treatments A, B and C_W (plants with adequate watering during cultivation) in I_{-25} and b were tested for the single species and N treatments with one-way ANOVA for unbalanced data (GLM program; SAS 6.04). This was necessary because the sizes of the data sets were unequal (in A and B, $n = 8$; in C_W, $n = 4$). The variances of the single data sets did not differ significantly (tested by the method of Cochran; see Sachs, 1984); (3) the effects of species, N supply and water regime on I_{-25} and b were tested for the freezing treatment C ($C_W + C_D$) with three-way ANOVA (SAS 6.04). The analyses of variance were followed by Tukey's test. The significance level was $P < 0.05$ in each case.

RESULTS

Growth and nutrient concentrations

During July, a second flush was generated by nearly all oaks supplied with 'luxurious N' but only in approx. 60 % of the oaks grown with 'normal N'. In the 'luxurious N' treatment, the relative increment in shoot length was also significantly higher (sessile oak, 25.5 ± 3.0 %; pedunculate oak, 40.8 ± 4.6 %) than in the trees grown with 'normal N' (sessile oak, 14.5 ± 1.4 %; pedunculate oak, 21.8 ± 2.0 %), and the number of leaves also increased. In comparison with the adequately watered oaks, the drought-stressed trees showed diminished increments in shoot length. However, no attempt was made to test these differences for significance because of the small number of plants subjected to the drought treatment. In the 'luxurious N' treatment, the leaf discoloration started later than in the 'normal N' treatment in autumn; in spring, in those plants that had not been harvested, the leaves sprouted earlier. In spring, new shoots developed in all of the remaining plants that had been kept outside.

In the oaks supplied luxuriously with N, the N concentrations of leaves and bark were distinctly higher and the C : N ratios were lower than in the trees with normal N supply (Table 1, Fig. 1). The foliar N concentrations of the trees grown with 'normal N' were generally within the range 19–26 mg g^{-1} d. wt, which was determined as the normal range for young pedunculate and sessile oaks kept outside; within this range, growth is medium to good (van den Burg, 1985). The foliar N concentrations of oaks supplied with 'luxurious N' were above that range (Table 1, Fig. 1). The K concentrations were within the normal range and did not differ between the N treatments (Table 1). However, the oaks that had been luxuriously supplied with N exhibited significantly increased N : K ratios owing

Table 1. *Concentrations of N, K and Mg and ratios N : K and N : Mg in the leaves of 2-yr-old* Quercus petraea *and* Q. robur *grown with normal or 'luxurious' N supply, and subjected to adequate watering or to drought stress*

Species and N supply	*n*	Nutrient concentrations (mg g^{-1} d. wt) and ratios (g g^{-1})				
		N	K	N : K	Mg	N : Mg
Quercus petraea, adequately watered						
N normal	50	21.8 ± 0.3	8.10 ± 0.21	2.8 ± 0.1	3.50 ± 0.09*	6.4 ± 0.2
N luxurious	48	29.7 ± 0.4*	8.10 ± 0.19	3.8 ± 0.1*	3.00 ± 0.10	10.4 ± 0.4*
Quercus petraea, drought-stressed						
N normal	4	21.1 ± 0.7	8.03 ± 0.76	2.7 ± 0.4	3.96 ± 0.43*	5.5 ± 0.6
N luxurious	4	32.3 ± 2.2*	9.15 ± 0.79	3.6 ± 0.1*	2.77 ± 0.15	11.9 ± 1.4*
Quercus robur, adequately watered						
N normal	50	23.6 ± 0.5	8.51 ± 0.23	2.9 ± 0.1	3.75 ± 0.10*	6.5 ± 0.2
N luxurious	48	29.7 ± 0.5*	8.87 ± 0.24	3.5 ± 0.1*	3.11 ± 0.16	10.4 ± 0.5*
Quercus robur, drought-stressed						
N normal	4	23.5 ± 1.2	11.10 ± 1.20	2.2 ± 0.3	3.60 ± 0.20	6.7 ± 0.7
N luxurious	4	34.1 ± 2.1*	8.92 ± 0.72	3.9 ± 0.3*	3.72 ± 0.17	9.3 ± 0.9*

n, number of plants. *, significantly higher than for oaks grown with different N supply within a given species and watering treatment.

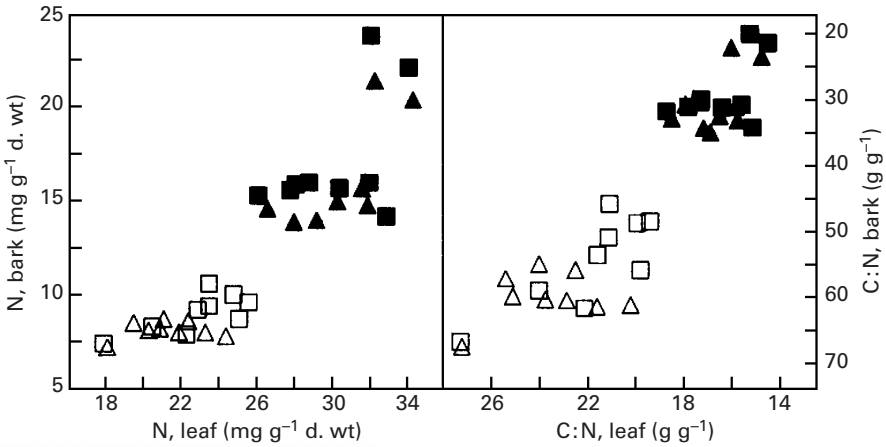

Fig. 1. Nitrogen concentrations and C : N ratios of leaves and bark from sessile oaks (triangles) and pedunculate oaks (squares) grown with normal (open symbols) or 'luxurious' N supply (closed symbols). The values of the bark result from combined samples made for the various freezing treatments or harvest dates. The values of the leaves are the means of the respective data sets.

CAMBRIDGE UNIVERSITY PRESS

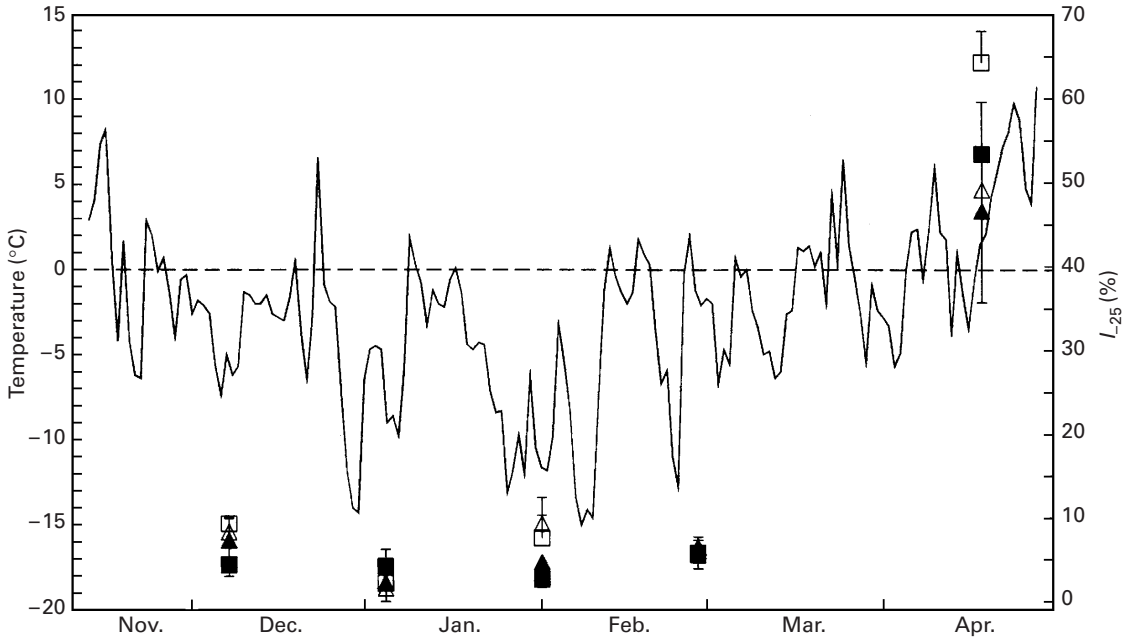

Fig. 2. Daily minimum air temperature during winter and early spring (solid line), and indices of injury determined after artificial freezing at −25°C (I_{-25}) of bark tissue from sessile oaks (triangles) and pedunculate oaks (squares) grown with normal (open symbols) or 'luxurious' N supply (closed symbols). The broken line marks 0°C.

to their higher N concentrations. In all N treatments, the Mg concentrations exceeded the normal range but were significantly lower in the 'luxurious N' treatment than in the 'normal N' one (except for the drought-stressed pedunculate oaks). Together with the elevated N concentrations, this resulted in significantly higher N:Mg ratios in the leaves of these trees.

Frost hardiness of outside plants

As early as mid-November, the minimum air temperatures sank below 0°C, and remained low until the end of March except for a few short periods (Fig. 2). These subzero temperatures were reflected in the I_{-25} values, which were low from December to the end of February. The I_{-25} values did not increase noticeably before April, when the minimum temperatures generally exceeded 0°C. On none of the dates of measurement were significant differences found between the species and N treatments (Fig. 2). From two previous findings, it can be concluded that the I_{-25} values indicate the actual frost hardiness of the plants. First, curves of I_t values, which were constructed to assess the frost hardiness of the bark and were obtained from several freezing temperatures applied to the tissue (−5 to −30°C), differed significantly only when the I_{-25} values of those curves also exhibited a distinct difference (Thomas *et al.*, 1996). Second, a good correlation was found between I_t values determined for bark tissue of adult oaks with temperatures between −20 and −30°C on the one hand, and the temperature course during winter, on the other (Thomas & Blank, 1996). Thus,

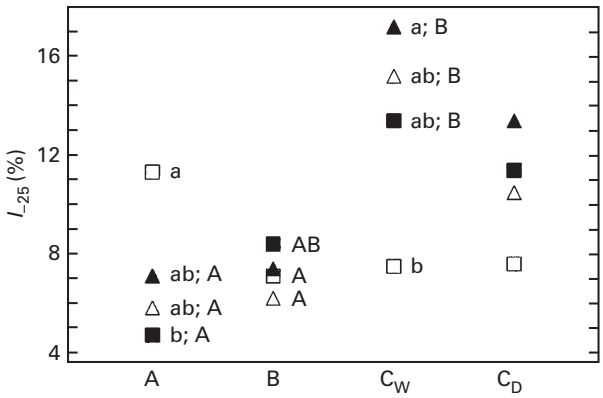

Fig. 3. Indices of injury determined after artificial freezing at −25°C (I_{-25}) of bark tissue from sessile oaks (triangles) and pedunculate oaks (squares) grown with normal (open symbols) or 'luxurious' N supply (closed symbols). Before the freezing tests of the bark tissue, the entire plants had been subjected to freezing for 10 d (treatment A), 21 d (B), or two periods of 4 d interrupted by a frost-free period of 8 d (C). The plants of the last group were subdivided into one that had been adequately watered (C_W) and one that had been subjected to drought stress (C_D) during the preceding growing season. Values marked with different letters differ significantly (one-way ANOVA). Lower-case letters represent the results of comparisons within the individual freezing treatments A, B, C_W and C_D; capital letters indicate the results of comparisons between the freezing treatments of the adequately watered plants (A, B and C_W) conducted for the single species and N treatments.

the similar I_{-25} values of the outside plants investigated in this study during winter indicate that all oaks possessed a similar level of frost hardiness at the start of the various freezing treatments, and that they could be compared by statistical analyses in spite of different dates of the treatments.

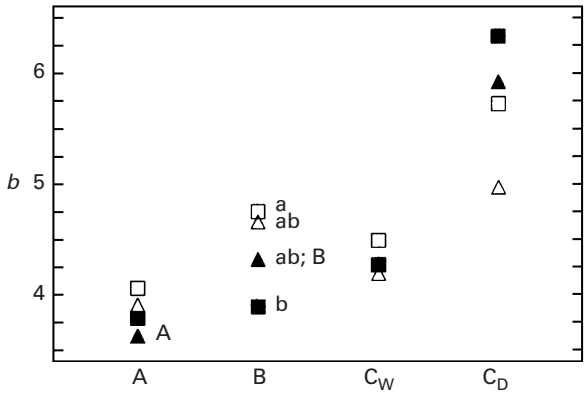

Fig. 4. Slopes (b) of the regression RC $= a + b \ln(t)$ (change of the relative conductivity (RC, %) with the logarithm of time (t, h)) obtained from bark tissue of oaks subjected to different freezing treatments. For symbols and treatments, see the legend to Fig. 3.

Frost hardiness and freezing damage after freezing treatments

The I_{-25} values of oaks that had been luxuriously supplied with N were not higher than in oaks of the same species with a normal N supply after the freezing treatments (Fig. 3). On the contrary, after the 10 d treatment, the normally nourished pedunculate oaks exhibited a higher I_{-25} than those grown with luxurious N. Except for the normally nourished pedunculate oaks, the freeze–thaw treatment of adequately watered plants resulted in significantly higher I_{-25} than did continuous freezing. After the freeze–thaw treatment, the sessile oaks showed a tendency towards higher I_{-25} values than the pedunculate oaks.

The oaks supplied with 'luxurious N' also failed to show higher rates of electrolyte leakage than the plants grown with 'normal N' (Fig. 4). On the contrary, after 21 d of treatment, the normally nourished pedunculate oaks exhibited higher b values than those grown with 'luxurious N'. In the adequately watered plants, the different freezing treatments did not result in different b values except in the sessile oaks grown with 'luxurious N', which showed a significantly higher leakage rate after the 21 d treatment than plants cooled for only 10 d. Oaks that had been subjected to drought stress during summer exhibited higher leakage rates than adequately watered trees.

In Table 2, the results of the three-way analysis of variance performed for the oaks that were treated with the freeze–thaw cycle (treatment C) are summarized. The total model yielded a significant F-value only in the case of the electrolyte leakage rates. Here, the factor WATERING led to a significant difference between the data sets by increasing the b values in drought-stressed trees compared with adequately watered plants (Fig. 4). All the other factors and factor combinations did not result in significant effects. With respect to the I_{-25} values, the factor SPECIES accounted for a significant difference between the data sets: the sessile oaks exhibited higher values (i.e. a lower frost hardiness) than the pedunculate oaks (Fig. 3). The F value of the factor N-SUPPLY almost reached the threshold of significance: the oaks grown with luxurious N tended to show higher I_{-25} values (Fig. 3). However, the F value of the total model derived from the I_{-25} values was insignificant.

Even when the entire data sets from oaks kept outside and oaks subjected to the various freezing treatments were considered, no significant correlations were found between the N concentrations or the C:N ratios of the bark, on the one hand, and the I_{-25} or b values, on the other.

Table 2. *Results of the three-way analysis of variance and subsequent Tukey's test, which were performed on the oaks of the freezing treatment C (freeze–thaw cycle), for the total model and the independent variables SPECIES (sessile or pedunculate oak), WATERING (adequately watered or drought stress) and N-SUPPLY (normal or 'luxurious')*

		I_{-25}		b	
Factor	df	F value	P	F value	P
Model	7	1.84	0.125	4.27*	0.003
SPECIES	1	5.14*	0.033	1.53	0.228
WATERING	1	2.08	0.162	23.92*	0.0001
N-SUPPLY	1	4.16	0.053	1.49	0.234
SPECIES × WATERING	1	0.86	0.362	0.56	0.463
SPECIES × N-SUPPLY	1	0.46	0.503	0.31	0.585
WATERING × N-SUPPLY	1	0.03	0.864	2.07	0.163
SPECIES × WATERING × N-SUPPLY	1	0.17	0.686	0.00	0.992

The dependent variable was frost hardiness (I_{-25}) or freezing damage (b). Total $n = 32$; four observations per species, watering and N supply. *, significant at $P < 0.05$.

CAMBRIDGE UNIVERSITY PRESS

Table 3. *Results of the TTC tests on bark and buds of 2-yr-old sessile oaks* (Quercus petraea) *and pedunculate oaks* (Q. robur), *grown with different N supplies, after different freezing treatments*

	Freezing treatment				
				C (20 d; freeze–thaw)	
Species and N supply	Control	A (10 d)	B (21 d)	+water (C_W)	−water (C_D)
Quercus petraea					
N normal	++/++	+/++	+/(−)	(+)/−	(+)/−
N luxurious	++/++	+/++	+/(−)	(−)/−	(−)/−
Quercus robur					
N normal	++/++	++/++	+/+	(+)/−	(+)/−
N luxurious	++/++	++/++	+/+	(+)/−	(+)/−

Control, plants kept outside during the entire winter; +water, adequately watered; −water, drought-stressed during the preceding growing season. Bark/buds: ++, bright red (vital); +, red to purple (vital); (+), weakly to patchy red (less vital); (−), weakly and patchy red to brownish (subvital; in buds, insertion to the stem red but leaf primordium brown); −, brown (dead).

The results obtained from the bark samples by the TTC test (Table 3) confirm the findings determined with the electrolyte leakage methods. In particular, there is a good correlation with the indices of injury. After freezing, the bark of oaks supplied with 'luxurious N' did not show more severe damage than samples from trees grown with 'normal N', except in the sessile oaks subjected to the freeze–thaw treatment. In these trees, the bark was assessed as 'subvital'. This agrees with the increased I_{-25} values of those trees (Fig. 3). The bark samples were less vital after the freeze–thaw treatment than after continuous freezing. However, an effect of the water supply during cultivation was not observed. Again, this agrees well with the I_{-25} values (Fig. 3). Buds of the sessile oak also proved to be more susceptible to freezing than those of the pedunculate oak. The buds of the sessile oak were subvital after prolonged continuous freezing, whereas those of the pedunculate oak were still vital (Table 3). The freeze–thaw treatment killed the buds in all of the oaks, independent of species, N-supply and watering during cultivation. In the control plants, which were kept outdoors throughout the entire winter and not subjected to artificial freezing, bark and buds were vital on all dates.

DISCUSSION

In studies on the frost hardiness or freezing damage to woody shoots, the measurement of the relative conductivity after a fixed time interval and the determination of the electrolyte leakage rate provided similar results (Deans *et al.*, 1995; Sheppard *et al.*, 1995). A good correlation was also found on comparing the results of the electrolyte leakage rate with the results of a visual assessment of damage (Caporn *et al.*, 1994). In our investigation, the agreement between the TTC test and the computed I_{-25} values was slightly better than that between the TTC test and the slopes of the electrolyte leakage rate. The reason for this might be the relatively high surface : volume ratio of the bark samples, which was probably responsible for the large efflux rates of electrolytes into the solution, and might have prevented more distinct differences between the treatments.

From December to the end of February, during the period of deep winter frost, the average I_{-25} values of the bark from the oaks that were kept outside were always $<10\,\%$ (Fig. 2). In spring, none of the remaining oaks exhibited symptoms of freezing damage. Moreover, all of the bark samples taken from the plants kept outside were assessed as vital with the TTC test (Table 3, Control). These findings indicate that freezing conditions resulting in I_{-25} values $<10\,\%$ do not lead to freezing damage to the bark of the oaks. By contrast, if the I_{-25} value exceeded $15\,\%$, as with the sessile oaks grown with luxurious N and subjected to the C_W treatment, the bark tissue was subvital according to the TTC test (Fig. 3, Table 3). Thus, we conclude that the vitality of bark tissue from frost-exposed indigenous oak species is noticeably decreased if an I_{-25} of $15\,\%$ (as determined under the selected conditions) is exceeded. Accordingly, for twigs of *Fraxinus americana* subjected to the same test of frost hardiness, freezing temperatures resulting in an index of injury of $15\,\%$ were regarded as 'killing temperatures' (Alexander *et al.*, 1984). For twigs of *Quercus rubra*, temperatures yielding an index of injury of $10\,\%$ were regarded as critical (Flint, 1972).

The low I_{-25} values for bark samples taken from outside plants between the beginning of December and the end of February (Fig. 2) indicate that the trees had completed their frost-hardening process before the start of the artificial freezing treatments. Values for I_{-25} of $<10\,\%$ were also determined in

bark samples of mature sessile oaks during periods of complete frost hardening, whereas samples from dehardened trees had values of $>50\,\%$ (Thomas & Blank, 1996). In the present study, values of that magnitude were obtained in April, after a distinct increase in the minimum air temperature. These results represent an additional corroboration of the method and indicate its suitability for quantifying the state of frost hardiness in plant tissues (Alexander *et al*., 1984; Burr *et al*., 1990).

With regard to the effect of excess N on the frost hardiness or freezing damage of bark tissue, both methods relying on electrolyte leakage yielded similar results: a negative effect of low C : N ratios of the bark tissue on its frost hardiness was not found. This finding is in contrast to the results obtained with bark samples from mature oaks (Thomas & Blank, 1996). The reason for this might be the different ages of the plants. The frost sensitivity of seedlings and young plants can differ from that of mature ones (Sakai & Larcher, 1987). Other possible reasons are site factors and the course of the temperature during winter. The influence of site factors cannot be quantified, whereas the time course of the temperature during the investigation periods offers a satisfying explanation. In the earlier investigation (Thomas & Blank, 1996), the sampling dates for which significant differences between oaks of different N status were found had been preceded by relatively warm periods. Here, decreased C : N ratios in the bark tissue might have led to a partial loss of frost hardiness. By contrast, in the present study, the temperatures were low and the frost hardiness was high during the entire period of frost-hardiness investigation. The high degree of frost hardiness might therefore have prevented any adverse effects due to excess N.

In other investigations, different effects of increased N supply on frost hardiness have been found. Investigations on deciduous forest trees are scarce. In the woody shrub *Calluna vulgaris*, N fertilization raised the frost tolerance (Caporn *et al*., 1994). Many more studies have been conducted on conifers. In *Pinus sylvestris*, the frost hardiness of shoots was decreased by previous N fertilization (Aronsson, 1980) and by exposure to NH_3 (Dueck *et al*., 1990/91). In both cases, the N treatments had led to significant increases in foliar N concentrations. In *Picea abies*, N fertilization did not affect the frost hardiness of the needles (Wiemken *et al*., 1996). In *Picea rubens*, N fertilization even increased the frost hardiness of the needles, which exhibited significantly higher N concentrations after the N treatment (DeHayes *et al*., 1989; L'Hirondelle *et al*., 1992). However, the N status of the plants before the treatments exerts a great influence on the extent and direction of the alteration in frost hardiness. Misting of *Picea rubens* seedlings with N-containing solution had no effect on the frost hardiness of the needles if the plants had been sufficiently supplied with nutrients before the treatment; however, freezing injury to nutrient-deficient seedlings was significantly decreased by the misting treatment (Klein *et al*., 1989). Corresponding results were obtained from *Pseudotsuga menziesii*: the frost hardiness was decreased at both low and high foliar N concentrations, and was highest just below the level that was correlated with optimal growth (Larsen, 1978). These different reactions of frost hardiness to the N supply probably reflect the physiological effect of N. The amino acids arginine and proline act not only as storage compounds of N but are also assumed to contribute to the protection against freezing injury (Sakai & Larcher, 1987). Hence, it is to be expected that plants with a low N supply are more prone to frost damage. By contrast, excess uptake of N, particularly of NH_4^+, increases the demand for carbon skeletons owing to the need for enhanced N assimilation. This could lead to decreased contents of that fraction of carbohydrates and their derivatives that serves in cryoprotection (soluble sugars and cyclitols). In the present study, frost hardiness and freezing damage were largely unchanged in bark and buds of a given oak species, although foliar N concentrations were distinctly above normal. Indications of a decrease in the frost hardiness by high N supply were found only in the visual assessment of bark samples from sessile oaks that might have been partly dehardened during a freeze–thaw cycle. It is therefore concluded that excess N as a single factor does not affect the frost hardiness of bark tissue of pedunculate and sessile oaks if they are at the level of full frost hardening. With regard to other nutrients, K in particular is important for frost hardiness, because its supply increases frost hardiness and can compensate for the adverse effect of excess N (Larcher, 1985). Although the foliar N : K ratios of the oaks grown with luxurious N supply were slightly above the normal range (which is approx. 1.5 to 3 according to the respective element concentrations given by van den Burg, 1985), they were not sufficiently unbalanced to interfere with the level of frost hardiness.

The frost hardiness of plants differs at the level of the various organs. Usually, buds are less frost-resistant than woody stems (Sakai & Larcher, 1987). In *Quercus petraea*, dormant buds are frost-resistant at temperatures down to -24°C, and in *Q. robur* down to -27°C (Till, 1956). This is consistent with our observation that buds of *Q. petraea*, but not those of *Q. robur*, were damaged by freezing at -25°C for 21 d. However, the N status of the plants had no effect on the frost hardiness of the buds. By contrast, buds of *Picea rubens* bear a higher risk of frost injury when the foliar N concentrations are elevated, even if the frost hardiness of the needles remains unaffected (L'Hirondelle *et al*., 1992; Hadley *et al*., 1993).

CAMBRIDGE UNIVERSITY PRESS

The water supply during the preceding vegetation period and the type of freezing treatment had a distinctly greater influence on the frost hardiness of bark and buds than the N supply. Oaks that had been subjected to drought stress exhibited significantly increased leakage rates after the freeze–thaw cycle. Prolonged drought can decrease frost resistance by decreasing carbon gain (Sakai & Larcher, 1987). In certain regions of northern Germany where increased damage to oak had occurred in the mid-1980s, severe winter frost, which is regarded as a synchronizing factor for the onset of the damage, was preceded by summer drought (Hartmann & Blank, 1993). However, in the affected stands, severe insect defoliation did occur simultaneously; the effects of the single stress factors can therefore not be clearly separated (Hartmann & Blank, 1993). Nevertheless, repeated insect defoliation, severe winter frost and summer drought are regarded as the primary causal factors that can lead to increased damage to oak, but at least two of these factors have to occur simultaneously to trigger oak decline (Hartmann, 1996).

In winter, the degree of frost hardiness is dependent on the actual temperature and the state of dormancy. In late winter, an increase in temperature dehardens the plants to a greater extent than in midwinter, at the state of deep dormancy (Sakai & Larcher, 1987). This might explain the differences in the sensitivity of frost hardiness to elevated N contents between the mature oaks (Thomas & Blank, 1996), in which a decrease in frost hardiness was found in bark tissue with low C:N after mild periods, and the seedlings (this study), which had experienced prolonged freezing temperatures from late December to March. It also explains the severe impact of the freeze–thaw treatment, which was performed in March, on the frost hardiness and freezing damage. At that time, the tissues were probably no longer at the state of deep dormancy; the 8 d period of above-zero temperatures might have led to a significant dehardening; the oaks were therefore more susceptible to freezing damage during the subsequent freezing.

According to the TTC test and the I_{-25} values, the sessile oak tended to be more sensitive to the freezing treatments. This agrees well with a previous investigation on bark tissue of adult oaks (Thomas & Blank, 1996) and is in accordance with the commonly held assessment of sessile oak being more susceptible to winter frost than pedunculate oak (Ellenberg, 1996), as well as with the fact that the distribution of the pedunculate oak extends much farther into the continental regions of Europe than that of the sessile oak (Meusel *et al*., 1965).

In summary, a distinct effect of excess N on the frost hardiness and freezing injury of above-ground parts of oak was found only in combination with a freeze–thaw treatment, in which the plants had presumably dehardened during the frost-free period, and only by visual assessment (TTC test) of bark from the sessile oak. The duration of the frost stress and the course of the temperature (as was shown by the TTC test and the indices of injury) as well as drought stress during the preceding growing season (demonstrated by the electrolyte leakage rates) had a larger impact on freezing injury than the N treatment. We conclude that the supply of excess N does not significantly affect the midwinter frost hardiness of mature pedunculate and sessile oaks, if they are fully hardened. The results suggest that the freezing damage to oaks in the mid-1980s was caused by extreme midwinter frosts and was not exacerbated by N deposition.

ACKNOWLEDGEMENTS

We thank Professor Dr Wolfgang Schmidt and Karl-Heinz Obal (Institut für Waldbau, Fakultät für Forstwissenschaften und Waldökologie, Universität Göttingen, Göttingen, Germany), for providing and maintaining the freezing cabinet.

REFERENCES

Alexander NL, Flint HL, Hammer PA. 1984. Variation in cold-hardiness of *Fraxinus americana* stem tissue according to geographic origin. *Ecology* **65**: 1087–1092.

Aronsson A. 1980. Frost hardiness in Scots pine (*Pinus silvestris* L.). II. Hardiness during winter and spring in young trees of different mineral nutrient status. *Studia Forestalia Suecica* **155**: 1–27.

Baumeister W, Ernst W. 1978. *Mineralstoffe und Pflanzenwachstum*, *3rd edn*. Stuttgart, Germany: G. Fischer Verlag.

Burr KE, Tinus RW, Wallner SJ, King RM. 1990. Comparison of three cold hardiness tests for conifer seedlings. *Tree Physiology* **6**: 351–369.

Caporn SJM, Risager M, Lee JA. 1994. Effect of nitrogen supply on frost hardiness in *Calluna vulgaris* (L.) Hull. *New Phytologist* **128**: 461–468.

Deans JD, Billington HL, Harvey FJ. 1995. Assessment of frost damage to leafless stem tissues of *Quercus petraea*: a reappraisal of the method of relative conductivity. *Forestry* **68**: 25–34.

DeHayes DH, Ingle MA, Waite CE. 1989. Nitrogen fertilization enhances cold tolerance of red spruce seedlings. *Canadian Journal of Forest Research* **19**: 1037–1043.

Dueck TA, Dorèl FG, Ter Horst R, van der Eerden LJ. 1990/91. Effects of ammonia, ammonium sulphate and sulphur dioxide on the frost sensitivity of Scots pine (*Pinus sylvestris* L.). *Water, Air and Soil Pollution* **54**: 35–49.

Ellenberg H. 1996. *Vegetation Mitteleuropas mit den Alpen*, *5th edn*. Stuttgart, Germany: Ulmer Verlag.

Flint HL. 1972. Cold hardiness of twigs of *Quercus rubra* L. as a function of geographic origin. *Ecology* **53**: 1163–1170.

Flint HL, Boyce BR, Beattie DJ. 1967. Index of injury – a useful expression of freezing injury to plant tissues as determined by the electrolytic method. *Canadian Journal of Plant Science* **47**: 229–230.

Hadley JL, Amundson RG, Laurence JA, Kohut RJ. 1993. Red spruce bud mortality at Whiteface Mountain, New York. *Canadian Journal of Botany* **71**: 827–833.

Hartmann G. 1996. Ursache des 'Eichensterbens' in Deutschland – Versuch einer Synthese bisheriger Befunde. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* **318**: 125–151.

Hartmann G, Blank R. 1993. Etiology of oak decline in northern Germany. History, symptoms, biotic and climatic predisposition, pathology. In: Luisi N, Lerario P, Vannini A, eds. *Recent advances in studies on oak decline*. Bari, Italy: Dipartimento di Patologia vegetale, 277–284.

Klein RM, Perkins TD, Myers HL. 1989. Nutrient status and

winter hardiness of red spruce foliage. *Canadian Journal of Forest Research* **19**: 754–758.

Kolb TE, Steiner KC, Barbour HF. 1985. Seasonal and genetic variations in loblolly pine cold tolerance. *Forest Science* **31**: 926–932.

Larcher W. 1969. Anwendung und Zuverlässigkeit der Tetrazoliummethode zur Feststellung von Schäden in pflanzlichen Geweben. *Mikroskopie* **25**: 207–218.

Larcher W. 1985. Frostresistenz. In: Rademacher B, Richter H, eds. *Handbuch der Pflanzenkrankheiten*, *7th edn.*, vol. 1, part 5. Berlin: Parey Verlag, 177–259.

Larsen JB. 1978. Die Frostresistenz von 60 verschiedenen Douglasien-Herkünften sowie über den Einfluss der Nährstoffversorgung auf die Frostresistenz der Douglasie. In: Larsen JB, Muhle O, Lohbeck H. *Untersuchungen zur Bestandesbegründung der Douglasie. Schriften aus der Forstlichen Fakultät der Universität Göttingen und der Niedersächsischen Forstlichen Versuchsanstalt* **52**: 1–126.

Levitt J. 1980. *Responses of plants to environmental stresses*, *2nd edn.*, vol. 1: *Chilling*, *freezing*, *and high temperature stresses*. New York, USA: Academic Press.

L'Hirondelle SJ, Jacobson JS, Lassoie JP. 1992. Acidic mist and nitrogen fertilization effects on growth, nitrate reductase activity, gas exchange, and frost hardiness of red spruce seedlings. *New Phytologist* **121**: 611–622.

Meusel H, Jäger E, Weinert G. 1965. *Vergleichende Chorologie der zentraleuropäischen Flora*. Jena, Germany: VEB G. Fischer Verlag.

Murray MB, Cape JN, Fowler D. 1989. Quantification of frost damage in plant tissues by rates of electrolyte leakage. *New Phytologist* **113**: 307–311.

Ritchie GA. 1991. Measuring cold hardiness. In: Lassoie JP, Hinckley TM, eds. *Techniques and approaches in forest tree ecophysiology*. Boca Raton, FL, USA: CRC Press, 557–582.

Sachs L. 1984. *Applied statistics – a handbook of techniques*, *2nd edn*. New York, USA: Springer Verlag.

Sakai A, Larcher W. 1987. *Frost survival of plants*. (Ecological Studies, vol. 62.) Berlin, Germany: Springer Verlag.

Schlag MG. 1994. Das europäische 'Eichensterben' und seine Ursachen – von einem phytopathologischen Standpunkt aus gesehen. *Centralblatt für das gesamte Forstwesen* **111**: 243–266.

Shapiro SS, Wilk MB. 1965. An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591–611.

Sheppard LJ, Franssen I, Cape JN. 1995. Frost hardiness of Norway spruce treated with acid mist. Evaluation of the electrolyte leakage rate technique. *Environmental and Experimental Botany* **35**: 139–149.

Soikkeli S, Kärenlampi L. 1984. The effects of nitrogen fertilization on the ultrastructure of mesophyll cells of conifer needles in northern Finland. *European Journal of Forest Pathology* **14**: 129–136.

Thomas FM, Blank R. 1996. The effect of excess nitrogen and of insect defoliation on the frost hardiness of bark tissue of adult oaks. *Annales des Sciences Forestières* **53**: 395–406.

Thomas FM, Büttner G. 1993. Excess nitrogen, drought, and winter frost as possible predisposing factors of oak decline in northern Germany. In: Luisi N, Lerario P, Vannini A, eds. *Recent advances in studies on oak decline*. Bari, Italy: Dipartimento di Patologia vegetale, 285–291.

Thomas FM, Kiehne U. 1995. The nitrogen status of oak stands in northern Germany and its role in oak decline. In: Nilsson LO, Hüttl RF, Johansson UT, eds. *Nutrient uptake and cycling in forest ecosystems*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 671–676.

Thomas FM, Blank R, Hartmann G. 1996. Der Einfluss von Stammexposition, Stickstoff-Status und Blattfrass auf die Frosthärte des Bastes von Alteichen. *Verhandlungen der Gesellschaft für Ökologie* **26**: 153–160.

Till O. 1956. Über die Frosthärte von Pflanzen sommergrüner Laubwälder. *Flora* **143**: 499–542.

van den Burg J. 1985. *Foliar analysis for determination of tree nutrient status – a compilation of literature data*. (Rapport No. 414.) Wageningen, The Netherlands: Rijksinstituut voor Onderzoek in de Bos- en Landschapsbouw 'De Dorschkamp'.

van den Driessche R. 1976. Prediction of cold hardiness in Douglas fir seedlings by index of injury and conductivity method. *Canadian Journal of Forest Research* **6**: 511–515.

Wiemken V, Kossatz L, Ineichen K. 1996. Frost hardiness of Norway spruce grown under elevated atmospheric CO_2 and increased nitrogen fertilizing. *Journal of Plant Physiology* **149**: 433–438.

CAMBRIDGE UNIVERSITY PRESS