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Effect of Fertilisation on the Development of Freezing Tolerance in Silver Birch (*Betula pendula* Roth.) and Blue Holly (*Ilex* × *meserveae* S.Y. Hu 'Blue Princess')

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Summary

Swedish ornamental nurseries are supplying fertiliser to field crops only during the first half of the growing season, due to the concern of growers that late fertilisation will delay development of freezing tolerance. Effects of fertilisation on freezing tolerance were investigated in two climate chamber experiments. Three different fertilising strategies were used on plants of silver birch and blue holly; one treatment according to ordinary practice with all fertiliser applied within the first half of the growing season, secondly a treatment with all fertiliser almost evenly spread out over the growing season, and the third treatment as the second one but with additional potassium and phosphorus added at the end of the growing season. The total applied amount of nitrogen was the same for all treatments. The plants had a growing season of 12 weeks, followed by 6 weeks of autumn climate, simulated in the climate chamber. During the autumn period plants were tested for freezing tolerance in freezing tests. The study showed that a fertiliser strategy with an almost even distribution of a balanced fertiliser during the growing season had no negative effect on the development of frost tolerance in silver birch and blue holly. Growth and freezing tolerance showed small differences between treatments, although the rate of development of freezing tolerance seemed to be slightly faster in the third treatment. However, the results showed differences in growth and development of freezing tolerance between the experiments, indicating that other growth factors than nutrient distribution had an impact on developing freezing tolerance. Nevertheless, the results indicate that growers can even out the application of fertilisers over the entire growing season without jeopardising the development of freezing tolerance and, thus, decrease the risk for nutrient leakage to underlying soil and water table.

Key words. Fertiliser strategy – frost tolerance – hardiness development – *Betula pendula* – *Ilex* × *meserveae* 'Blue Princess'

Introduction

The strategy, that Swedish nurseries are supplying fertiliser to their field crops only during the first half of the growing season, is based on practical experience and has its origin in concern of nursery operators that late fertilisation, especially with nitrogen, will delay development of bud dormancy and, thus delay development of stress tolerance. The major reason for this concern is that the nurseries lift their field stock during autumn, store it in cold storage during winter and sell or replant in spring. The importance of lifting date and storage time has been investigated previously (Lindqvist 2001; Lindqvist and Bornman 2002; Lindqvist and Asp 2002), and the most important factor for achieving high quality after storage is that lifting does not occur before plants have developed a certain level of stress tolerance. The common practice in Swedish ornamental nurseries is to apply the main bulk of fertiliser in spring, prior to planting, often as NPK with micronutrients. Additionally, calcium nitrate is applied once or twice during late spring and early summer, but not later than mid-July. The disadvantages of this strategy are the risk of nutrient leakage to the ground water table, especially with high precipitation during late spring or early summer, and the risk that plants will have low nutrient reserves in the autumn and, thus jeopardising growth and development the following spring.

Studies made on fertiliser effects on development of dormancy and cold acclimation are scarce and of very different experimental designs, thus giving equivocal results. Stimart and Goodman (1985) found that higher levels of nitrogen (N), applied as NH_4NO_3 during the growing season, had negative effect on cold acclimation in newly rooted cuttings of *Acer palmatum* Thunb. 'Bloodgood'. Rikala and Repo (1997) found that a high level of fertiliser, applied as NPK 11-4-25 from beginning of July to beginning of September, gave the highest level of frost hardiness in *Pinus sylvestris* L.

In *Eucalyptus globulus*, a high dose of N, applied during the growing season, improved development of hardiness, while no effect was found of high doses of phosphorus (P) or potassium (K) (Fernàndez et al. 2007). Dehayes et al. (1989) showed, in a trial with three different levels of N fertilisation with or without P, that moder-

ate increase of N fertilisation increased cold tolerance, while a higher level of N decreased it, while P did not influence cold tolerance at all in *Picea rubens* Sarg. JOZEFEK (1989) stated that high concentration of K reflected a low tolerance to frost in *Betula pendula* Roth. and JALKANEN et al. (1998) showed that deficit of K in *Picea sitchensis* increased frost hardiness. PERCIVAL et al. (1999) suggested that freezing tolerance in *Carpinus betulus* and *Populus alba* could be increased with supplementary application of calcium (Ca).

Controlled freezing test is a frequently used method for testing cold acclimation and frost hardiness. The method has shown to be in good agreement with field trials (BANNISTER 2003; TASCHLER and NEUNER 2004). The method involves exposing plants or plant parts to decreasing subzero temperatures down to a target temperature, which is held for a certain period followed by increasing temperatures. Unfortunately, a standard procedure of freezing tests has not been established, making it difficult to compare different experiments. The rate of decreasing temperatures is often 1–2 °C h^{-1}, which is presumed to mimic natural rates of cooling. The duration of exposure to the target temperature differs in different experiments, testing different species, from two to six hours (SAKAI and LARCHER 1987; VON FIRCKS 1992; STENER et al. 2002), followed by an increase of the temperature at a rate of 5 °C h^{-1}. To assess the result of the controlled freezing test, different methods are used such as visual assessment (FERNÀNDEZ et al. 2007), chlorophyll fluorescence (LENNARTSSON and ÖGREN 2002), digital image analysis (NEUNER and BUCHNER 1999) and electrolyte leakage (COLOMBO et al. 1995; STENER et al. 2002).

The aim of this study was to investigate, in a climate chamber, the effect of fertiliser application during the whole growing season, comparing it with normal nursery practice, and to test the development of cold hardiness by freezing test in two different species, *Betula pendula* Roth. and *Ilex* × *meserveae* S.Y.Hu 'Blue Princess'.

Materials and Methods

The study was carried out in two time separated experiments, the first experiment during the autumn 2007 and the second during spring 2008. Micropropagated plants of *Betula pendula* were used in both experiments and cutting propagated plants of *Ilex* × *meserveae* 'Blue Princess' were used in the second experiment.

Cultivation practice

In two experiments micropropagated birch plants (B14, Splendor White) were stimulated to elongate on WPM (Woody Plant Medium) based medium (LLOYD and MCCOWN 1980) with additional 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 0.001 mg l^{-1} α-naphthalene acetic acid (NAA) for 4 weeks kept in a climate chamber at 22 °C. The plants were then transplanted to a peat/perlite substrate (50/50 Vol %; Hasselfors S-jord) and rooted in the greenhouse for 4 weeks at 18 ± 2 °C. Finally the plants were transplanted to 3 L rose pots in a peat and sand mixture (Solmull, silversand 50/50 Vol %, limed with 0,035 kg grounded limestone per 30 L to a calculated pH of 5,5) and transferred to a climate chamber with a day length of 16 h, 20 °C day temperature and 15 °C night temperature. Plants were placed on trolleys with plates under the pots to ensure no water or nutrient loss. In the first experiment, the plant density was 36 plants m^{-2} and in the second experiment the density was 32 plants m^{-2}. The plants were provided with light from cool white fluorecent tubes (Sylvania; 330) at an intensity of 300 μmol m^{-2} s^{-1}. Directly after transplanting the substrate was watered to the maximal water holding capacity and fertiliser was added as a top dress.

The plants were watered as needed, with 100 ml at a time, in order not to drain the pots. Once a week the moisture in the substrate was checked with a commercial moisture meter and plants were watered until "wet" 7–9 on the 9 points scale if needed.

After 12 weeks of growth the climate programme in the chamber was changed to autumn with 15 °C day temperature and 5 °C night temperature and a day length of 8 h. Watering was reduced and adapted to the new conditions.

In the second experiment birch and Blue Holly were studied. Cuttings of *Ilex* × *meserveae* 'Blue Princess' from 6 year old field grown mother plants were stuck in a peat substrate mixed with perlite (50/50 Vol %; Hasselfors S-jord) and rooted in a mist chamber at 20 ± 2 °C for 6 weeks. The Holly plants were then transplanted to the same pot size and substrate as the birch plants and cultured under the same growing conditions.

Experimental set-up

The experiment was carried out in climate chambers in a phytotrone. Nutrients were added as a combined fertiliser (Yara, NPK 11-5-18 and PK 7-25) and as calcium nitrate (Yara, 15.5 % N). The content of macro nutrients in the commercial products are listed in Table 1. The same total amount of nutrients were added in all treatments, but in treatment 1 all nutrients were added in the first half of the growing season at 3 occasions, while in treatment 2 and 3 the nutrients were added at 6 different occasions. In treatment 3 the plants were fertilised also with additional PK at the 2 last occasions, which is commonly used in fruit orchards to improve ripening and quality of the fruit. Fertilisers added in the three treatments during the trial period are presented in Table 2.

Table 1. Macro nutrient content in the commercial fertilisers used in the experiments, given as percentage of weight.

	NPK 11-5-18	$Ca(NO_3)_2$	PK 7-25
Nitrogen	11.0	15.5	–
Phophorus	5.0	–	7.0
Potassium	18.0	–	25.0
Magnesium	1.6	–	–
Calcium	1.0	18.8	9.3
Sulphur	10.0	–	3.8

Table 2. Amount of fertiliser added during the course of the experiment, given as gram per pot as top dress.

Days in experiment	Treatment 1		Treatment 2		Treatment 3		
	NPK (g)*	$Ca(NO_3)_2$*(g)	NPK	$Ca(NO_3)_2$	NPK	$Ca(NO_3)_2$	PK (g)*
1	1.8		0.3	0.2	0.3	0.2	
16			0.3	0.2	0.3	0.2	
23		0.3					
30			0.3	0.1	0.3	0.1	
44		0.3	0.3	0.1	0.3	0.1	
58			0.3		0.3		0.33
72			0.3		0.3		0.33

* Fertilizer doses were given in gram per pot as top dress

Measurements and analysis

Shoot length. Shoot length in birch was measured from the substrate surface to the shoot tip at 6 and 5 different occasions until the end of the growing season. In holly the total height was only measured at the beginning and at the end of the trial.

Nutrient availability in the soil fluids. Three liquid suction lysimeters per pot were placed in three pots of each treatment for the sampling of soil water to monitor nutrient availability. At four different occasions, always one week after fertilization and one day after irrigation, soil water was collected and analysed for macronutrients.

Freezing trial. Frost hardiness was evaluated by measuring the electrolyte leakage after controlled freezing according to STENER et al. 2002. For the freezing tests programmable freezers connected to a PC were used by courtesy of SkogForsk, the Swedish Forestry Institute, in Ekebo, Sweden. Two freezing programs were used to achieve the target freezing temperatures of −5 °C and −15 °C, respectively which was kept for six hours. The temperature dropped 2 °C hour^{-1} and increased after freezing with 5 °C hour^{-1}.

For the freezing test, shoot samples were taken every week from the second to the sixth week of the artificial autumn period in the first experiment and from the first to the sixth week in the second experiment. The leading shoot from each plant was cut in 6 node pieces starting 8 to 10 nodes below the apical bud. The node pieces were put into plastic bags and moist was added with a spray bottle. The bags were sealed and kept at 0 °C until the start of the freezing experiment. Stem segments from all treatment were kept as control in plastic bags in the fridge at +4 °C.

Electrolyte leakage assessment. After the freezing treatment the stem segments were cut on both sides keeping the middle 1 cm pieces which were put in 10 ml ultra pure water (Millipore) in plastic tubes sealed to avoid evaporation and contamination. The samples were put on a shaker for 24 h before the first measurement with a conductivity meter (EcoScan CON5, Eutech instruments) was made. Then the samples were autoclaved for 20 min in order to cause total cell death. After shaking the samples for another 24 h the conductivity was measured again and the index of injury was calculated according to FLINT et al. (1967), as follows:

Eq. 1 $$RC_{frozen} = EC_{frozen}/EC_{frozen + autoclaved}$$

Eq. 2 $$RC_{control} = EC_{unfrozen}/EC_{unfrozen + autoclaved}$$

Eq. 3 $$I = \frac{100(RC_{frozen} - RC_{control})}{1 - RC_{control}}$$

where: EC = electric conductivity of elute
RC = relative conductivity
I = index of injury

The index of injury can thus be seen as method for expressing freezing injury by converting percentage release of electrolytes to a scale where the unfrozen sample is given a value of zero and the autoclaved sample a value of 100 (FLINT et al. 1967).

Statistical analysis

All data were statistically analysed with ANOVA and were tested for equal variances, using Minitab Statistical Software (release 15.1). The data of freezing test from −15 °C were, furthermore analysed in a non-linear regression, using SAS Statistical Software (PROC NLIN). The data from the different treatments were fitted to the following model with F-test ($P \leq 0.05$):

Eq. 4 $$f(I) = C + \frac{D - C}{1 + \exp(a + b \cdot I)}$$

where: D = upper limit of the curve
C = lower limit of the curve
a = describes the location of the curve
b = describes the slope of the curve

Results

The nutrient concentration in soil water of the two experiments is shown in Table 3. Birch plants consumed all

Table 3. Nitrogen (NO_3 and NH_4), phosphorus, potassium and calcium concentrations in soil fluid during the course of experiment.

Trial	Treatment	Days in experiment	Nutrients in the soil fluid (mg/l)				
			NO_3-N	NH_4-N	P	K	Ca
Birch Exp. 1	1	8	109.4	74.4	41.2	184.7	165.3
		36	132.7	9.6	27.6	244.3	335.3
		64	5.6	0.1	1.7	18.3	41.8
		78	0.2	0.1	0.5	9.6	54.1
	2	8	43.3	10.3	4.9	31.1	65.5
		36	32.8	1.9	4.5	49.0	119.7
		64	4.6	1.7	6.8	43.5	56.3
		78	0.5	0.1	1.3	29.3	98.8
	3	8	46.0	9.8	4.2	31.0	65.5
		36	69.3	1.0	2.6	46.8	216.3
		64	0.9	0.1	25.2	76.5	65.5
		78	0.6	0.1	28.6	129.7	183.1
Birch Exp. 2	1	8	122.0	146.0	94.7	386.5	191.0
		36	79.3	0.1	11.2	167.0	169.0
		64	0.1	0.1	0.9	74.2	107.8
		78	0.1	0.1	0.7	87.4	203.0
	2	8	29.8	19.9	9.3	58.8	72.2
		36	0.1	0.1	0.7	29.9	86.6
		64	0.1	0.1	1.5	74.6	218.0
		78	0.1	0.1	0.7	51.8	142.0
	3	8	34.6	19.0	6.9	44.5	66.8
		36	17.6	4.9	2.2	61.3	140.0
		64	0.1	0.1	13.3	127.5	324.0
		78	0.1	2.4	20.1	165.0	260.5
Holly Exp. 2	1	8	34.6	46.8	25.4	154.0	98.7
		36	107.0	3.8	9.6	139.0	145.5
		64	48.2	0.1	4.4	114.4	91.7
		78	10.2	1.0	2.7	107.0	74.6
	2	8	29.1	14.5	5.4	37.2	62.0
		36	86.5	14.0	26.8	82.7	130.0
		64	71.0	3.4	19.8	148.0	175.5
		78	71.8	3.9	19.2	173.0	199.0
	3	8	10.1	14.0	5.3	31.7	48.6
		36	59.1	8.2	7.8	64.6	98.8
		64	31.8	0.8	21.3	140.5	111.1
		78	42.1	2.5	31.9	219.5	153.5

soluble N, independent of the treatment, but the content of N in the soil water was clearly higher at the 2 first sampling occasions in treatment 1 than in treatment 2 and 3. In treatment 1 and 2 also all P were consumed, while treatment 3, due to the extra PK application, showed a small increase towards the end of the trial period. Though more difficult to interpret, it seems clear that K and Ca declined during the trial period in treatment 1, while it was equal, though fluctuating, or increasing in treatment 2 and 3. For the holly plants, only treatment 1 consumed most of the soluble N and P, while treatments 2 and 3 had a steady level or a slight increase over the trial period. Potassium and Ca decreased in treatment 1 and increased in treatments 2 and 3 over the trial period (Table 3).

The growth in both species showed none or small differences between the treatments. In birch, there was a significant difference between treatment 1 and 2 at the final measurement in the first trial, but the actual differences were small. However, the results showed a large difference in growth between the two trials for the birch plants. The first trial had on average 13 cm higher growth than the second trial (Fig. 1). The holly plants showed no difference in growth between treatments and had an average growth increment of ca. 17 cm (data not shown).

The most commonly used method of grading frost tolerance in an electrolyte leakage assessment is the LT_{50} level, used to assess different species specific frost tolerance and, thus, their possible geographical distribution (Taschler and Neuner 2004). The LT_{50} value is defined as an index of injury of 50 %, when using several freezers covering a span of minimum temperatures and plotting the index of injury values against minimum temperatures. This method is not applicable in this case as the aim of this study was to follow the development of frost tolerance from start of autumn climate and further on. For this purpose an injury level of 10 %, I_{10}, was introduced, which represents a level of no or small injuries, and as such gives an assessment of how well and how fast the plants are developing frost resistance. Applied on the results from this study the results showed that both species, in the second trial, were frost resistant for the −5 °C freezing level after two weeks (Fig. 2). In the first trial, however, the birch plants did not reach this level until after four weeks. The different treatments reached the I_{10}-level approximately at the same time in the second trial, but a trend of faster development of frost tolerance could be seen for treatments 2 and 3 in the first trial (Fig. 2).

A clear difference was found between the species in the −15 °C freezer, where the holly plants developed frost tolerance much faster than birch plants (Fig. 3). The holly plants reached the I_{10}-level after 5 weeks of short photoperiod and lower temperatures, while birch plants did not reach this level during the 6 weeks test period. In the non-linear regression analysis, the data from treatment 2 of the holly plants had to be excluded from the analysis, as the results clearly deviated at the 2 first measuring points and, thus, did not fit the model (Fig. 3). The differences between treatments were small, however the non-linear regression showed a significant difference, where treatment 3 had a steeper slope compared to treatment 1 and 2 in birch and treatment 3 had a steeper slope compared to treatment 1 in holly. However, the steeper slope in treatment 3 had little actual effect as the development of frost tolerance started later in treatment 3, at least in the second experiment (Fig. 3).

Discussion

The higher level of soluble N in the soil water in treatment 1 was expected, considering the strategy of fertilis-

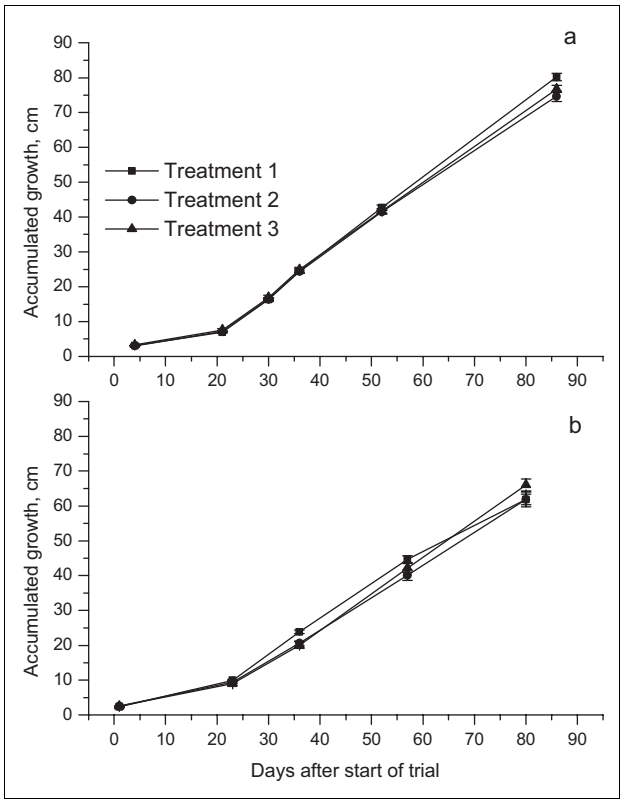

Fig. 1. Accumulated growth in birch (*Betula pendula* Roth.) during two different experiments, (a) autumn 2007 and (b) spring 2008, for 3 different fertilising strategies. Treatment 1: all nitrogen applied during the first half of the growing season at 3 occasions, treatment 2: nitrogen applied every 2 weeks over the growing season and treatment 3: nitrogen applied every 2 weeks over the growing season with additional application of PK at the two last occasions. Error bars represent standard error of means.

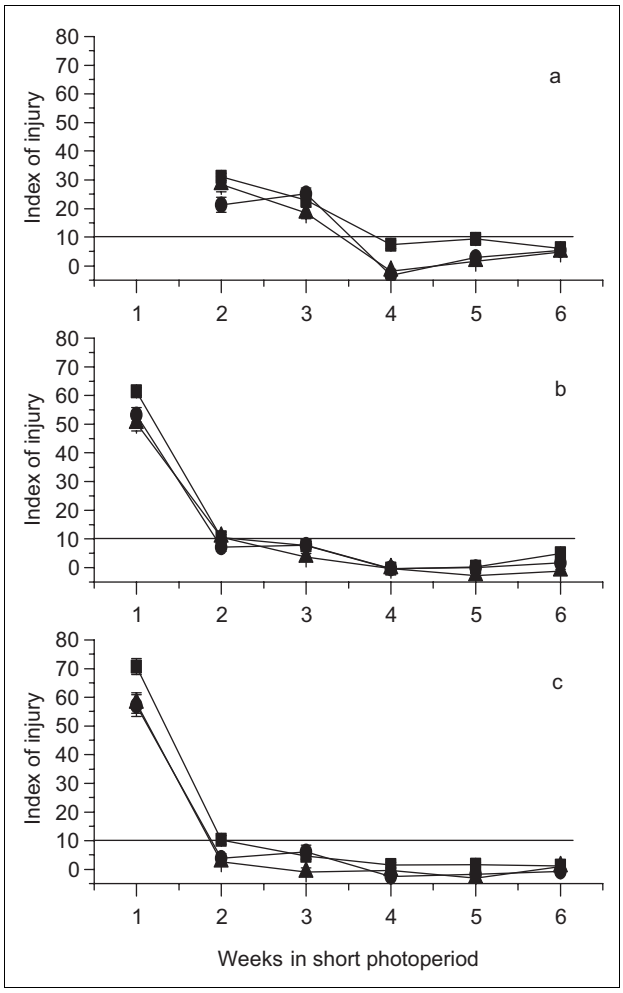

Fig. 2. Frost resistance to −5 °C in silver birch (*B. pendula* Roth.), (a) experiment 1 and (b) experiment 2, and (c) blue holly (*I. meserveae* S.Y. Hu), measured as index of injury of electric conductivity in freezing test, for 3 different fertilising strategies. Treatment 1: all nitrogen applied during the first half of the growing season at 3 occasions, treatment 2: nitrogen applied every 2 weeks over the growing season and treatment 3: nitrogen applied every 2 weeks over the growing season with additional application of PK at the two last occasions. Horizontal line represents index of injury 10, which represents no or small damage. Error bars represent standard error of means.

ing. It showed, however, the higher potential risk of leakage of soluble N from the soil in a situation with higher precipitation. Furthermore, as all soluble N was consumed by the birch plants, or bound to soil particles, over the growth period in all treatments, a situation with higher precipitation could have created an N shortage in plants in treatment 1, leading to plants with too low N levels and, thus, jeopardising subsequent growth and development. (Table 2).

Controlled freezing tests are, as mentioned before, in good agreement with field trials and, thus, the result of this study showed that a fertiliser strategy with an almost even distribution of a balanced fertiliser during the growing season had no negative effect on the development of frost tolerance in silver birch and blue holly. This result shows similarities with the results of DEHAYES et al. (1989) and SUNDHEIM FLØISTAD (2002). However, looking at birch plants in the 2 different trials, it was clear that a more vigorous growth gave a slower development of frost resistance, at least down to −5 °C (Fig. 1 and 2), independent of fertiliser strategy. It is, however, difficult to explain the difference in growth between the trials. The only detectable difference was that a larger amount of birch plants were used in the first trial than in the second, which gave a denser stand and, probably, a stronger competition for light. Furthermore, in the second experiment, half of the climate chamber was used for the holly plants, which grow much slower and thus do not compete for light. Nevertheless, it seems, in this case, to be a negative correlation between growth and development of frost resistance, though not affected by fertiliser strategy.

An additional application of P and K towards the end of the growing season did neither increase nor decrease the development of frost tolerance at the end of the trial period. However, looking at the non-linear regression for the −15 °C freezing test, additional application of P and K showed a steeper slope and, thus, a faster development of frost tolerance (Fig. 3). DEHAYES et al. (1989) showed that

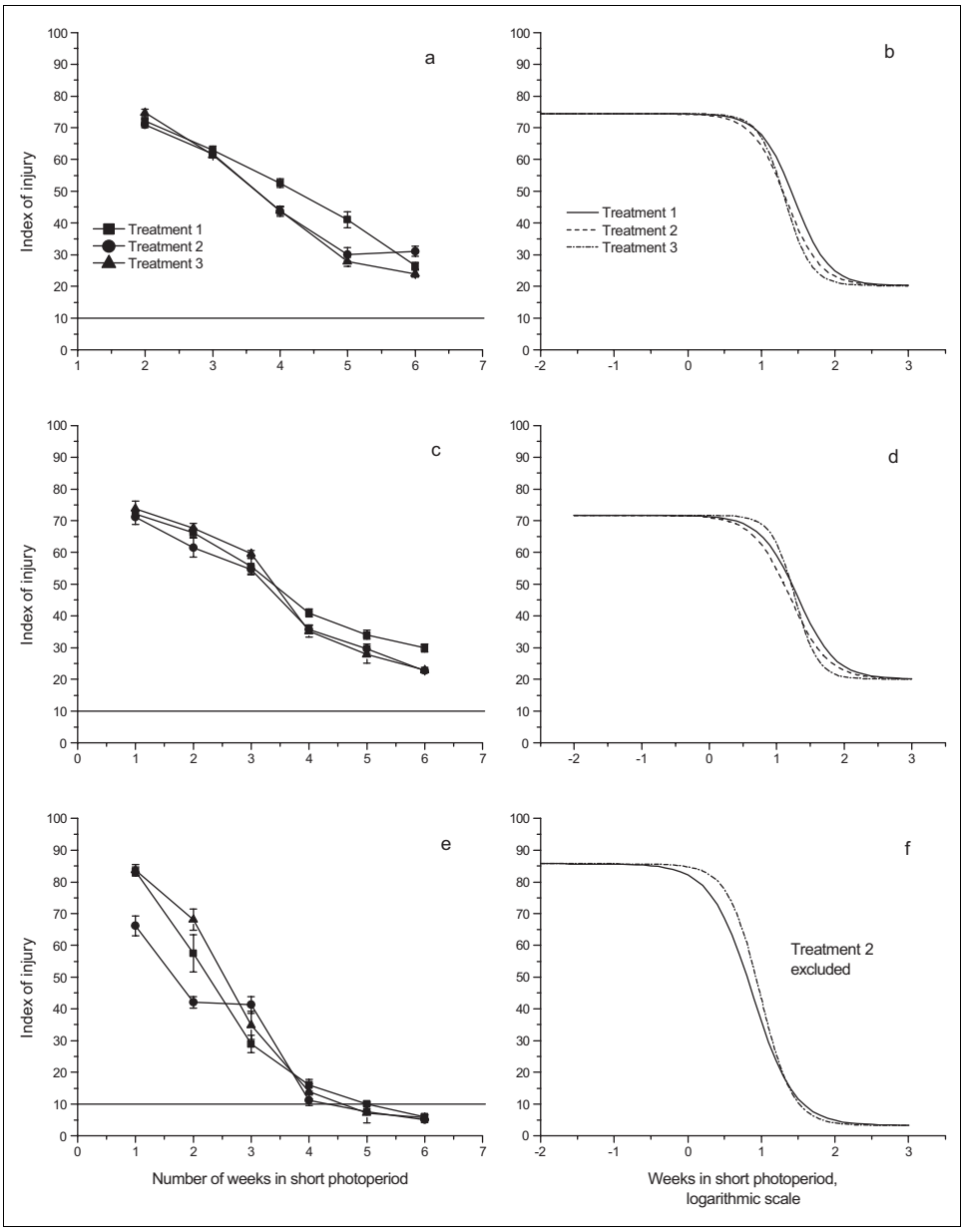

Fig. 3. Development of frost resistance and its non-linear regression down to −15 °C in (a, b) silver birch (*B. pendula* Roth.), in experiment 1, and (c, d), experiment 2 and (e, f), blue holly (*I. meserveae* S.Y. Hu), measured as index of injury of electric conductivity in freezing test, for 3 different fertilising strategies. Treatment 1: all nitrogen applied during the first half of the growing season at 3 occasions, treatment 2: nitrogen applied every 2 weeks over the growing season and treatment 3: nitrogen applied every 2 weeks over the growing season with additional application of PK at the two last occasions. Error bars in figure a, c and e, represents standard error of means. Horizontal line, in (a), (c) and (e), represents index of injury 10, which represents no or small damage.

P fertilisation did not affect development of freezing tolerance in *Picea rubens*. However, the results from these trials contradict the results of Jozefek (1989), who stated that high concentration of K gave a low tolerance to frost in *Betula pendula*. It also contradicts the results of Jalkanen et al. (1998), who showed that deficit of K in *Picea sitchensis* increased frost hardiness. It is not possible, in this case, to conclude which of the nutrients that accelerated the development of frost tolerance or if it is the combination as such. The fertiliser used, PK 7-25, contains, besides P and K also 9.3 % Ca and, as mentioned earlier, supplementary application of Ca has been shown to increase frost tolerance in *Carpinus betulus* and *Populus alba* (Percival et al. 1999).

The results of this study showed that a balanced fertiliser, applied as NPK 11-5-18 with micro nutrients, over the entire growth period, did not negatively affect development of frost resistance in silver birch and blue holly. To avoid leakage of nutrients, primarily N, and to have well-nourished plants at the end of the growing season, nursery operators should evenly distribute the application of fertiliser over the entire growing season.

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