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There is No Direct Relationship between N-status and Frost Hardiness in Needles of NH_3-Exposed Scots Pine Seedlings

By

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Summary

CLEMENT J.M.A.M., DE BOER M., VENEMA J.H. & VAN HASSELT P.R. 2000. There is no direct relationship between N-status and frost hardiness in needles of NH_3-exposed Scots pine seedlings. - Phyton (Horn, Austria) 40 (3): (21) - (33).

The effect of short-term atmospheric ammonia deposition on frost hardening of needles of three-month-old seedlings of Scots pine *(Pinus sylvestris* L.) was studied. Plants were frost hardened under short day and moderate temperature conditions in the laboratory during exposure to gaseous NH_3 concentrations of 400 or 1000 nl l^{-1} for 4 to 6 weeks. Exposure to NH_3 resulted in an increase of free ammonium and nitrogen content of the needles. Soluble sugar and starch content were not affected. Photosynthetic capacity and chlorophyll a concentrations were increased as a consequence of NH_3 exposure, but chlorophyll b and carotenoid were not influenced. NH_3 exposure did not decrease frost tolerance of the needles. Exposure to 1000 nl l^{-1} NH_3 even resulted in an increase of frost hardiness. It was concluded that frost tolerance of Scots pine seedlings is not negatively affected by the alterations of N-status upon short-term NH_3 exposure.

Introduction

Air pollution with ammonia is a serious environmental problem in many parts of Western Europe, especially in The Netherlands. Considerable amounts of ammonia are volatized in the air due to a concentration of intensive livestock industry in certain parts of the country. In these areas the mean annual NH_3 concentration range from 25-40 nl l^{-1}, with peak concentrations of 350-500 nl l^{-1} (ADEMA 1987, BLEEKER & ABEN 1994). Close to sources of emission the

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atmospheric ammonia deposition consist for 70% of dry (gaseous NH_3 + NH_4^+ aerosols) and 30% of wet (NH_4^+) deposition (ERISMAN & HEIJ 1991). The dry deposition taken up is mainly absorbed by the shoot, while the wet deposition is absorbed by the root/mycorrhizal system (PÉREZ-SOBA & al. 1994a). Air pollution with ammonia has been shown to severely affect plant and ecosystem functioning (VAN DER EERDEN 1992, PÉREZ-SOBA 1995) and is considered to be one of the main reasons for the observed forest decline in The Netherlands (VAN BREEMEN & al. 1982, ROELOFS & al. 1985, VAN DIJK & ROELOFS 1988).

The increased sensitivity of plants to natural stress factors is believed to be one of the factors responsible for the negative effects of NH_3 pollution on plants (NIHLGÅRD 1985). It was shown that frost sensitivity can be enhanced by atmospheric NH_3. VAN DER EERDEN 1982 observed an increased frost sensitivity in Brassica species upon exposure to NH_3. Also Corsican pine (*Pinus nigra* var. maritima) and Scots pine (*Pinus sylvestris* L.) were more damaged by frost when exposed to NH_3 (VAN DER EERDEN 1982, DE TEMMERMAN & al. 1988, DUECK & al. 1991). This negative effect on frost tolerance was attributed to the nitrogen fertilization by ammonia which prolonged growth in autumn and slowed down metabolic processes associated with frost hardening (HUTTUNEN & al. 1981, FRIEDLAND & al. 1984, DUECK & al. 1991). Another explanation was given by VAN DER EERDEN 1982 who hypothesized that an increase of free ammonium, originating from NH_3 uptake by the leaves, could lead to a saturation of membrane lipids and consequently result in an increased frost sensitivity. Besides, it was supposed that the metabolization of ammonia could lead to a shortage of carbohydrates, which in turn could decrease carbohydrate associated frost tolerance (VAN DER EERDEN 1982, FRIEDLAND & al. 1984, DUECK & al. 1991). However, until now it is still unclear which factor actually plays a role in the increased frost sensitivity in the presence of atmospheric ammonia.

The purpose of this study was to investigate whether exposure to atmospheric NH_3 lead to changes in ammonium, nitrogen and carbohydrate content and if such changes were related to an increase of frost sensitivity of Pinus sylvestris needles. Until now, the increased frost sensitivity of Scots pine was found mainly after rather long-term NH_3 exposures with concentrations near or slightly above mean annual NH_3 concentrations of NH_3 polluted areas (DE TEMMERMAN & al. 1988, DUECK & al. 1991). However under field conditions short time periods of peak concentrations occur during spreading of animal manure. Recently it was shown that short-term exposure to relatively high concentrations of atmospheric ammonia does not affect frost hardening of three- and five-year-old Scots pine trees (CLEMENT & al. 1999). In this study it was investigated if the frost tolerance of young, three months old Scots pine seedlings can be affected by short-term exposures to high NH_3 concentrations of 400 and 1000 nl l^{-1} for 6 weeks.

Materials and Methods

Scots pine seedlings were raised from seed obtained from the Voorsterbos seed garden

(National Forest Service, The Netherlands). Seeds were superficially sterilized for 60 min in 30% H_2O_2 (TRAPPE 1961) and sown in acid-washed river sand. After germination seedlings were transferred to plastic pots (0,15 l) filled with river sand (1 seedling per pot) and grown for 10 weeks in a greenhouse (day/night temperature 22/18°C; photoperiod 12 h, photon fluence rate minimal 200 µmol m^{-2} s^{-1} PAR; relative humidity 60-80%). Plants were watered twice a week with a 10 times diluted nutrient solution (CHRISTERSSON 1973). The other days demineralized water was given.

Three-week -old seedlings were exposed to NH_3 in 150 l stainless steel cabinets as described by CLEMENT & al. 1995. The desired ammonia concentration was achieved by adding pressurized ammonia (1000 µl l^{-1} NH_3 in N_2) to the incoming filtered airstream (charcoal filter G7XAD, element 1/1140X, Berko, Assen, The Netherlands) by mass flow controllers (ASM, Bilthoven, The Netherlands). The air exchange rate in the cabinet was 50 l min^{-1}. The air temperature in the cabinet was controlled by circulating cooling fluid through the double wall and bottom of the cabinet. Plants were exposed for 6 weeks to 0 (controls) and 400 nl l^{-1} NH_3 (experiment I) or to 0 and 1000 nl l^{-1} NH_3 (experiment II) at the first stage of hardening which consisted of a moderate temperature regime and short day conditions (day/night temperature 18/15°C, photoperiod 8 h, photon fluence rate 200-250 µmol m^{-2} s^{-1} PAR; relative humidity 50%). At biweekly intervals 12 fully grown needles were collected 1 hour after the beginning of the light period from 10 plants which had been exposed to 0 or to 1000 nl l^{-1} NH_3 (experiment II). Needles of the different trees were mixed and placed on ice. Half of the needles were used for determination of freezing tolerance. The remaining needles were used for determination of ammonium, sugar, starch, pigment and total nitrogen content. From the plants which had been exposed to 0 or to 400 nl l^{-1} NH_3 (experiment I) 60 needles (from 10 plants) were harvested at weekly intervals as described above and used for determination of the freezing tolerance.

For determination of free NH_4^+ content needles were washed three times with bidistilled water to remove adsorbed nitrogen, dried with tissue paper and placed on ice. For analysis of free ammonium content, 35-40 mg needle material (5 needles) was homogenized in 3 ml cold bidistilled water (adjusted to pH 3.0 with HCl) with a cooled pestle and mortar with addition of 10 mg Polyclar AT. The homogenate was centrifuged (20 min; 30000 g) at 4°C. The amount of free ammonium in the supernatant was determined with the phenol-hypochlorite method according to WEATHERBURN 1967. Measurements were made in triplicate.

For determination of total nitrogen content the needles were washed three times with bidistilled water to remove adsorbed nitrogen, dried with tissue paper, weighed and oven dried (48 h at 8°C). Total nitrogen content was determined on samples of 40-50 mg oven dried material (10 needles) with a modified Kjeldahl procedure (DONEEN 1932) to retain the nitrate during digestion. Samples were made in triplicate.

For determination of sugar and starch content the needles were immediately frozen in liquid nitrogen until analysis of the sugar and starch contents. Water-soluble sugars were extracted from 3 (15-20 mg FW) needles with 10 ml 96% ethanol, in 3 extraction steps. In the remaining residue, starch was hydrolized by boiling for 3 h with 20 ml 3% (v/v) HCl. Water-soluble sugar and starch content were determined colorimetrically with the anthrone reagent (FALES 1951) and expressed on a glucose basis. Samples and measurements were made in triplicate.

Photosynthesis was determined as oxygen production on samples of 5 needles of 1 plant, which had been exposed for 6 weeks either to 0 or to 400 nl l^{-1} NH_3. Needles were collected just before measurement at the middle of the light period.

Photosynthetic oxygen production was measured at 18°C in a temperature controlled leaf chamber with a Clark-type electrode (Model LD2, Hansatech, Kings Lynn, Norfolk, United Kingdom) at 2% oxygen and 4.5% CO_2 (DELIEU & WALKER 1981). Light response curves were made by recording oxygen evolution during 5 min at various photon flux densities up to 1400 µmol m^{-2} s^{-1}. Dark respiration was measured after a 10 min dark period. Measurements were done in duplicate for each NH_3 concentration.

Chlorophyll a, b, and total carotenoid contents were determined. Pigments from 5 needles (35-40 mg FW) per sample, which were cut in 3 mm pieces, were extracted in 5 ml 80% aceton

during 48 h at 4°C in the dark. After centrifugation (15 min; 3000 rpm) the absorbance of the supernatant was measured at 470, 646.8 and 663.2 nm respectively. Pigment content was calculated (LICHTENTHALER 1987). Measurements were done in triplicate.

For determination of freezing tolerance 50 needles from all sampled trees per NH_3 concentration were randomly taken and put in glass tubes, 5 needles per tube. The tubes were closed with a rubber stopper on which 1 cm^2 of moist filter paper was connected in order to maintain similar humidity in the tubes. Samples were placed in an ethanol filled cooling bath (Julabo F40, Julabo Labortechnik, GmbH, Seelbach, Germany), equilibrated at 4°C for 30 min and then frozen at a rate of 4°C h^{-1} to -25°C. To avoid supercooling of extracellular water, small ice crystals were added to the samples at a temperature of -1.5°C. Needle temperatures were measured with a copper/copper-nickel thermocouple which was positioned in one of the tubes. Samples were taken from the bath at different temperatures and stored overnight at 4°C in the dark. Control samples were stored at 4°C throughout the whole procedure. The next day, 1 ml of water was added to the tubes after which they were placed in the greenhouse (20/18°C, 14 h light, 50 µmol m^{-2} s^{-1}) for 2 days.

Frost tolerance of the needles was determined after 2 days of recovery with the chlorophyll fluorescence method (CLEMENT & VAN HASSELT 1996). Needles were dark adapted for 1 h after which minimal (F_O) and maximal (F_M) fluorescence were measured with a PAM fluorometer (PAM 101 and 103, Walz, GmbH, Effeltrich, Germany). The degree of frost hardiness was expressed as the lowest temperature, measured at intervals of 1°C, at which the ratio F_V/F_M (= $(F_M-F_O)/F_M$) was still equal to that of unfrozen, control samples.

Data were statistically tested by analysis of variance at $\alpha \leq 0.05$. All parameters were analysed using a two-way analysis of variance (ANOVA); results are presented in Table 1.

Results

Exposure of seedlings to 1000 nl l^{-1} NH_3 resulted in a significantly higher free ammonium concentration of the needles than in the control plants (Fig. 1, Table 1). In the NH_3 exposed plants, NH_4^+ concentration rapidly increased by a factor 3 during the first 2 weeks of the experiment, viz 152 versus 54 µg g FW^{-1}. After 2 weeks, NH_4^+ concentration decreased and after 6 weeks approximately the same value as at the beginning of the experiment was reached. In needles from the control plants, free ammonium gradually decreased throughout the whole period to a concentration of 14 µg g FW^{-1} at the end of the experiment (Fig. 1).

Total nitrogen content in the needles increased significantly upon exposure to 1000 nl l^{-1} NH_3 for 6 weeks from 14.2 mg g DW^{-1} to a content of 23.6 mg g DW^{-1}. In the needles from the control plants, total nitrogen content remained constant at a level of 14 mg g DW^{-1} (Fig. 2, Table 1). As a consequence, NH_3 exposed plants had a 54% higher nitrogen content at the end of the experiment.

Soluble sugar content of the needles increased during the 6 weeks of the experiment from 27 mg g FW^{-1} to 53 (control plants) or 49 (1000 nl l^{-1} NH_3) mg g FW^{-1}, respectively (Fig. 3A). At the same time starch content increased from 42 to 56 mg g FW^{-1} in the control plants and to 78 mg g FW^{-1} in the NH_3 exposed plants (Fig. 3B). Exposure to 1000 nl l^{-1} NH_3 did not affect both soluble sugar and starch contents significantly (Fig. 3, Table 1).

Photosynthesic capacity of seedlings which were exposed to 400 nl l^{-1} NH_3 for 6 weeks was significantly higher as in the control plants, viz 59 (400 nl l^{-1} NH_3)

versus 44 (controls) µmol g Chl^{-1} s^{-1} (Fig. 4). Quantum yield was not affected. Dark respiration was slightly higher in the NH_3 exposed plants as in the control plants, viz -1.9, respectively, -2.7 µmol O_2 g Chl^{-1} s^{-1}.

Chlorophyll a concentration was 20% higher in the NH_3 exposed plants compared to the controls, viz 1.46 respectively 1.19 mg g FW^{-1} (Fig. 5A). Chlorophyll b and carotenoid concentration were not affected significantly (Fig. 5B,C, Table 1).

Table 1. Effect of NH_3 exposure on free ammonium content, total nitrogen content, soluble sugar and starch content, photosynthesis, pigment content and freezing tolerance of needles of Scots pine seedlings. Results of a two-way ANOVA with exposure time, NH_3 concentration and their interacion as sources of variance: *P < 0.05; *** P < 0.001; ns, not significant; nd, not determined.

Dependent variable	Independent variable		
	Time	NH_3	Interaction
Total N content	***	***	***
NH_4^+	***	***	***
Soluble sugar	***	ns	ns
Starch	ns	ns	ns
Photosynthesis	nd	*	nd
Chl a	ns	*	ns
Chl b	***	ns	ns
Carotenoids	***	ns	ns
Freezing tolerance, 400 nl l^{-1}	***	ns	ns
Freezing tolerance, 1000 nl l^{-1}	***	*	ns

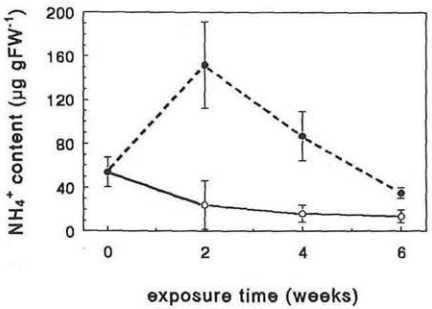

Fig. 1. Free ammonium content (µg g FW^{-1}) of needles of Scots pine seedlings exposed to 0 (o) and 1000 (•) nl l^{-1} NH_3 for 6 weeks at moderate temperature and short day conditions. Data represent means of 3 replicates ± SD.

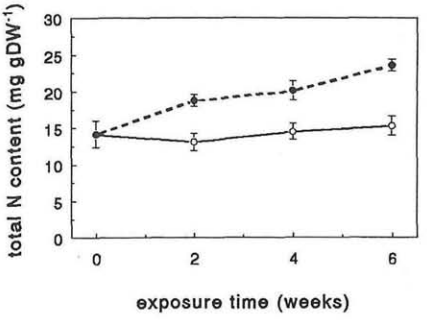

Fig. 2. Total nitrogen content (mg g DW^{-1}) of needles of Scots pine seedlings exposed to 0 (0) and 1000 (•) nl l^{-1} NH_3 for 6 weeks at moderate temperature and short day conditions. Data represent means of 3 replicates ± SD.

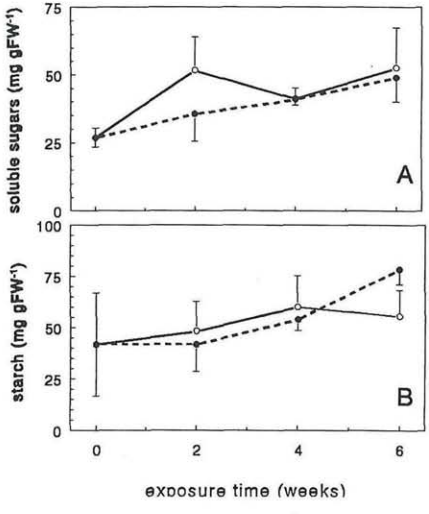

Fig. 3. Soluble sugars (A) and starch (B) content (mg g FW^{-1}) of needles of Scots pine seedlings exposed to 0 (o) and 1000 (•) nl l^{-1} NH_3 for 6 weeks at moderate temperature and short day conditions. Data represent means of 3 replicates ± SD.

Exposure of three-month-old Scots pine seedlings to short day conditions resulted in an increase of freezing tolerance of the needles (Fig. 6). At the beginning of the experiment, needles of plants exposed to 1000 nl l^{-1} NH_3 and their controls had a higher degree of freezing tolerance than the needles from the other plants, viz -7.8 °C versus -2.5 °C. However, the rate of hardening in the controls from both experiments was the same, about 2.5 °C per week resulting in the same absolute difference in freezing tolerance throughout the whole exposure time period of 4 weeks (Fig. 6).

Exposure to atmospheric NH_3 did not result in an increased frost sensitivity, neither in the plants exposed to 400 nor to 1000 nl l^{-1} NH_3 (Fig. 6). On the contrary, after 4 weeks seedlings exposed to 1000 nl l^{-1} NH_3 showed a significantly better frost hardening compared to the control plants, viz -18.9 °C compared with -16.0 °C (Fig. 6, Table 1).

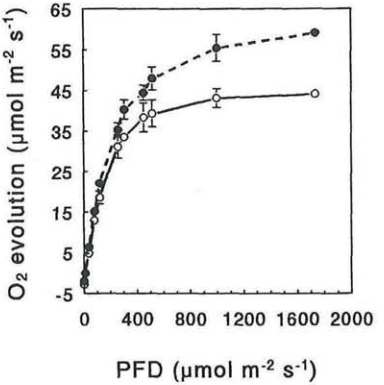

Fig. 4. Photosynthetic rate of 10 needles of three-month-old Scots pine seedlings measured as net O_2 production (µmol g Chl^{-1} s^{-1}) at different photon flux densities, after exposure for 6 weeks to 0 (o) and 400 (•) nl l^{-1} NH_3 at moderate temperature and short day conditions. Means of 3 measurements ± SD.

Discussion

Exposure of Scots pine seedlings to gaseous ammonia resulted in an increase in free ammonium and total nitrogen content of the needles, indicating that NH_3 was taken up by the needles and subsequently metabolized, as was also found in other studies with Scots pine (PÉREZ-SOBA & VAN DER EERDEN 1993, PÉREZ-SOBA & al. 1994a) and other plant species (COWLING & LOCKYER 1981, LOCKYER & WHITEHEAD 1986, GRUNDMANN & al. 1993). The uptake of gaseous ammonia occurs via the stomates and is mainly regulated by the stomatal resistance (VAN HOVE & al. 1987, 1991). Once inside the leaf, NH_3 is rapidly converted to NH_4^+, which can be metabolized to amino acids and proteins via the GS/GOGAT cycle (LEA & al. 1992). It was also shown in Pinus sylvestris needles exposed to NH_3, that the GS/GOGAT pathway was responsible for the incorporation of NH_3 (PÉREZ-SOBA & al. 1994a).

In the plants exposed to 1000 nl l^{-1} NH_3, free ammonium increased to a content of 152 µg g FW^{-1} after 2 weeks, whereafter it gradually decreased. This may be explained by an adaptation of the plant to the higher NH_3 level via an induction of GS (PÉREZ-SOBA & al. 1994a, b). During the first 2 weeks, GS activity may be too low to fully metabolize the nitrogen absorbed via the needles, leading

to an accumulation of free ammonium. Later, plants were possibly acclimated to the higher NH_3 level and the extra N input via an increased GS activity, which could result in a gradually lowering of the free ammonium content.
In the control plants, a gradual decrease of the NH_4^+ content was found with time. Also in 3-year-old Scots pine trees, ammonium content decreased with time (PÉREZ-SOBA 1990, PÉREZ-SOBA & al. 1994a). This decrease of the NH_4^+ content may be due to an increase of GS activity with increasing age, as was observed by PÉREZ-SOBA 1990, or may be a result of a higher N-incorporation or higher growth rate.

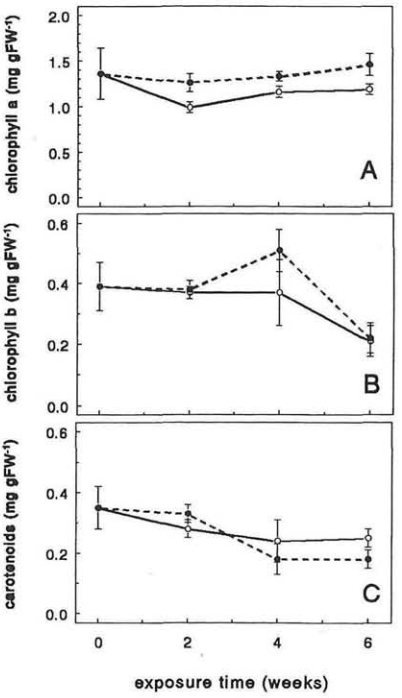

Fig. 5. Chlorophyll a (A), chlorophyll b (B) and carotenoid (C) content (mg g FW^{-1}) of needles of Scots pine seedlings exposed to 0 (o) and 1000 (•) nl l^{-1} NH_3 for 6 weeks at moderate temperature and short day conditions. Data represent means of 3 replicates ± SD.

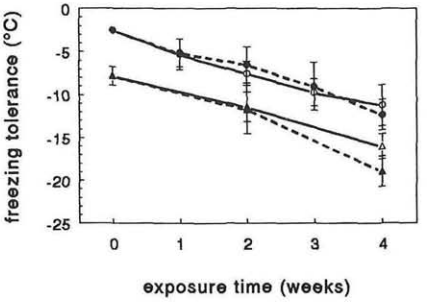

Fig. 6. Freezing tolerance (°C) of needles of Scots pine seedlings exposed to 0 (o) and 400 (•) or 0 (ç) and 1000 (Â) nl l^{-1} NH_3 for 4 weeks at moderate temperature and short day conditions. Data represent means of 5 replicates ± SD.

The accumulation of free ammonium in week 2 resulted in an internal concentration in the needles of 14 mM NH_4^+, which did not lead to any visible damage. On the other hand root growth of Scots pine seedlings was inhibited severely at comparable internal concentrations (VOLLBRECHT & al. 1989). Apparently needles of Scots pine are less sensitive to high NH_4^+ concentrations than roots. This may be due to the fact that needles of Scots pine were able to increase glutamine synthetase activity upon NH_3 exposure and subsequently incorporate the NH_4^+ into amino acids and proteins (PÉREZ-SOBA & al. 1994a), while NH_4Cl treated roots were not (VOLLBRECHT & al. 1989).

Total nitrogen content in the needles of the control trees was 1.4% on dry weight basis, which is a normal value for Scots pine needles. In the needles exposed to 1000 nl l^{-1} NH_3 nitrogen content increased to 2.4% after 6 weeks, a level far above the optimal level of 1.3-1.8% for Scots pine (HEINSDORF & KRAUß 1991). A high nitrogen content is often associated with a decrease of vitality, an increased sensitivity to natural stresses or with direct toxic symptoms (ARONSSON 1980, NIHLGÅRD 1985, BOXMAN & VAN DIJK 1988, DE TEMMERMAN & al. 1988, VAN DIJK & ROELOFS 1988). However, in this study, the high nitrogen content in the NH_3 exposed needles did not result in any direct toxic symptoms.

The incorporation of ammonium into amino acids has been shown to inhibit sucrose synthesis (CHAMPIGNY & al. 1992). However, in our study, ammonia assimilation was not coupled with a decrease in carbohydrate content of the NH_3 exposed needles. The fact that NH_3 exposure did not affect carbohydrate content may be explained by the higher rate of photosynthesis in these plants. The increased CO_2 assimilation possibly compensated for the extra carbon consumption by ammonia assimilation.

A stimulation of photosynthesis by atmospheric ammonia also found by others (VAN HOVE & al. 1991, VAN DER EERDEN & PÉREZ-SOBA 1992, VAN HOVE & BOSSEN 1994, CLEMENT & al. 1995) was correlated with a higher chlorophyll a content of the NH_3 exposed needles. At the same time chlorophyll b content of the

needles was not affected. As a result the chlorophyll a/b ratio of the NH_3 exposed needles was higher indicating an increase of the relative amount of photosystem II (ANDERSON & al. 1988, ANDERSON & CHOW 1992). In other studies with Scots pine and Douglas fir a stimulation of both chlorophyll a and b was observed upon NH_3 exposure (VAN HOVE & al. 1992, PÉREZ-SOBA & al. 1994a). Except for a relative higher photosystem II content, photosynthesis might have been increased due to a higher Rubisco content. In loblolly pine (*Pinus taeda* L.) seedlings and Scots pine seedlings the amount of Rubisco increased with higher nitrogen nutrition treatments and with higher nitrogen levels in the needles (GEZELIUS 1986, TISSUE & al. 1993).

Freezing tolerance of the needles was increased under conditions of moderate temperature and short day. In Scots pine these conditions induce the first phase of hardening (BERVAES & al. 1978). The differences in freezing tolerance between the experiments with 0 and 400 nl l^{-1} NH_3 and 0 and 1000 nl l^{-1} NH_3 may be explained by the time of the year the experiments were performed. Plants were exposed to 0 and 400 nl l^{-1} NH_3 from September until half October, while exposure to 0 and 1000 nl l^{-1} NH_3 took place from November until December. Although all plants were grown in the greenhouse under a 12 h additional light period at 200 µmol m^{-2} s^{-1} and the same temperature regime, plants grown in autumn were more frost hardened at the beginning of the experiment than summer grown plants. Apparently, outdoor conditions, like daylength, had a great impact on frost hardening of plants which were grown in the greenhouse. Nevertheless, it must be mentioned that the rate of hardening was not affected by the time of the year the experiments were performed, leading to the same absolute difference after 4 weeks.

From this study it became clear that frost sensitivity of Scots pine needles was not affected by the high free ammonium content in the NH_3 exposed seedlings, as suggested by VAN DER EERDEN 1982. Neither did this study give any indication of a shortage of carbohydrates as responsible for a reduced frost hardiness. Also, the high nitrogen content of the needles from the NH_3 exposed trees did not lead to a decreased frost hardiness of the needles. Frost tolerance was not affected or, in the case of 4 weeks exposure to 1000 nl l^{-1} NH_3, even increased. Similar results were found for red spruce (*Picea rubens* Sarg.), which also showed an enhanced frost tolerance upon nitrogen fertilization (DEHAYES & al. 1989, KLEIN & al. 1989). Apparently, frost tolerance is not directly related to the nitrogen content of the needles, as was suggested by others (HUTTUNEN & al. 1981, FRIEDLAND & al. 1984, DUECK & al. 1991). Besides, short-term exposure to high NH_3 concentrations did not lead to an increase of frost sensitivity of Scots pine needles, as it did in long-term exposure studies (DE TEMMERMAN 1988, DUECK & al. 1991, CLEMENT & al. 1996). Probably, the effect of NH_3 on frost sensitivity of Scots pine is a long-term, rather than a short-term effect, mediated by (an)other factor(s) than nitrogen content alone.

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