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Long-Term Symptoms Due to Metal and Acid Precipitation Treatments in Scots Pine (*Pinus sylvestris*) Needles in the Subarctic

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Abstract

In naturally harsh environments, such as arctic-alpine regions, even low levels of anthropogenic pollution appear to have detrimental impacts on plants. We studied the symptoms induced by metal and acid rain treatments in the ultrastructure of Scots pine (*Pinus sylvestris*) needles in one of the northernmost pine stands in the world at the Kevo Subarctic Research Station, Finland. Adult Scots pines at their natural growing site were irrigated during 1991–1996 with (1) water, (2) acid (pH 3), (3) copper and nickel (below CuNi), (4) Cu and Ni combined with acid, or (5) left unirrigated. The two youngest needle age classes were sampled in September 1996 for microscopy and elemental analyses, and the three youngest needle age classes for an evaluation of visible injuries. The CuNi treatment increased significantly both Ni and Cu concentrations. The accumulation of Cu in C + 1 needles was enhanced by acid addition. CuNi addition did not increase visible injuries, but significantly more ultrastructural dark accumulations and swollen thylakoids were seen in CuNi-treated C + 1 needles. The results show that, even in a seemingly harsh environment, pines are able to resist acid and metal-induced stress for a few years. However, tree growth is likely to suffer in the long run because the injuries seen in older needles suggest that the total capacity of the photosynthetic machinery is likely to decrease.

Introduction

Studies on the effects of acidification in manipulative experiments are often limited to only one or two growing seasons due to, for example, costs. Thus, long-term low-level pollutant exposure studies using adult forest trees at their natural growing sites are rare (but see Abrahamsen et al., 1994). A long-term acid and metal precipitation study of adult Scots pines (*Pinus sylvestris*) was started in 1991 at the Kevo Subarctic Research Station, Finland (69°45′N, 27°01′E) of the University of Turku. Kevo lies about 60 km north of the boreal coniferous forest zone, but the local river valleys contain some of the northernmost Scots pine stands in the world (Varmola, 1995). Even though the harsh climate restricts tree growth in Northern Lapland, the locally favorable microclimate due to the Gulf Stream allows pine growth (Varmola, 1995). On the other hand, under these stressful conditions, pines live at the limit of their ability to cope without suffering damage (Hustich, 1966). In spite of the fact that the ambient air pollution values are low at Kevo, e.g., the average estimated SO_2 concentration during July 1990–June 1991 was below 4 $\mu g\ m^{-3}$ (Laurila et al., 1991), episodes of high SO_2 concentrations may occur, especially during the winter (the maximum hourly average SO_2 concentration at Jäniskoski, 50 km east of Kevo, was 270 $\mu g\ m^{-3}$ in 1990 [Tuovinen et al., 1993]). The episodic high concentrations of metals and sulfur dioxide are caused by the emissions from the smelters on the Kola Peninsula in northwest Russia (Kryuchkov, 1993).

On the Kola Peninsula, the sulfur and heavy metals emitted from Monchegorsk smelters (68°N, 34°E) have been observed to decrease the frost hardiness of pine needles (Sutinen et al., 1996). Microscopic diagnostics of the damage to conifer needles caused by low temperatures at midboreal latitudes revealed separation of the plasma membrane from the cell wall and packing of the resulting space with electron-dense granules (Holopainen and Holopainen, 1988; Reinikainen and Huttunen, 1989). The alterations in the membraneous structures of cold-injured cells were most obvious. The net-like structure of the cytoplasm typical of winter-hardened needles was lost at an early stage of the winter injury (Reinikainen and Huttunen, 1989).

Acid rain, on the other hand, induced a decrease in the whole needle cross section, including the mesophyll, the phloem, and especially the xylem area (Bäck et al., 1994). Further, Bäck et al. (1994) observed increased vacuolation and lipid accumulations as a result of acid treatment. These symptoms, together with disintegration of the endoplasmic reticulum (Kukkola and Huttunen, 1998), and the poorly distinguishable thylakoids and chloroplast envelope were related to frost- and acid-induced injuries (Bäck et al., 1994).

Compared to acid rain, excessive deposition of metals might cause different diagnostic findings in the needle structure. Organic acids are important in chelating, for example, Ni and Zn (Lee et al., 1978; Yang et al., 1997), whereas Cu is associated with proteins and phenolic compounds (Neumann et al., 1995). Thus, in the present study, we aimed to determine whether different mechanisms for metal binding vs. acid addition could be identified in the damaged needle ultrastructure. Further, we tested the hypothesis that acid precipitation and heavy metal application would together increase winter injury by decreasing winter hardening in an environment where winter injuries are naturally a continuous threat. The sampling was done 6 yr after the baseline of the study in order to follow and describe the potential cumulative long-term symptoms.

Materials and Methods

EXPERIMENTAL DESIGN

The experiment performed at the Kevo site (69°45′N, 27°01′E) was designed using randomly selected plots in a natural mixed Scots pine (*Pinus sylvestris* L.) and mountain birch (*Betula pubescens* spp. *czerepanovii*, N.I. Orlova) forest. The monthly means of temperature and precipitation in the area are presented in Figure 1.

The experimental set-up consisted of five blocks, each of five plots. For each plot (within each block), one of the five treatments was

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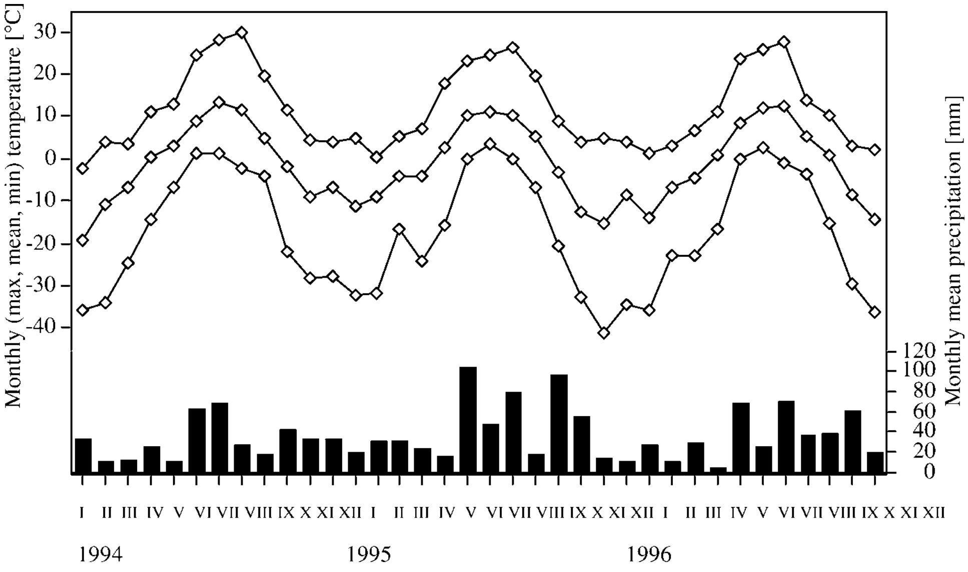

FIGURE 1. Monthly temperature (°C, maximum, mean and minimum) and precipitation (mm) at Kevo, Finland, during 1994–1996.

randomly allotted. Every plot contained one of the studied pines, making a total of 25 pines (five blocks, each containing five pines). The pines were 30 to 50 yr old and 1.8 to 5.3 m tall with a diameter of 1.6 to 8.2 cm at 1 m. The pines were exposed to Cu and Ni (pH 5.7, below referred to as CuNi) and/or acid (pH 3). The controls were unwatered dry controls (DC) and irrigated controls (IC), which had been sprayed with slightly acidified water (pH 5.6) to approximate ambient rain. Acidic water was prepared by adding H_2SO_4 to lake water, and the metals were added as sulfate salts in a water solution. The cumulative amounts of sulfur, copper, and nickel up to the sampling date are presented in Table 1. The irrigation treatments were applied by sprinklers over the canopy and the ground area of each plot twice a week. Each plot received a 5 mm dose of rain during each growing season (between June and August) during 1991–1996. The experimental design used here has been described in detail by Neuvonen and Suomela (1990).

SAMPLING AND MEASUREMENTS

Sampling

Soil samples were collected on 25 September 1996. The pH of both organic and mineral soil was measured from 0.01M KCl extract (soil:KCl = 1:3 vol). Needles for the elemental analyses (Table 2), evaluation of visible injuries (Table 3) and microscopic analyses (Table 4 and 5) were collected once on 25 September 1996 (the needles were C = 3 mo, C + 1 = 15 mo, or C + 2 = 27 mo old at the time of sample collection). Needles from one tree per plot and a total number of five pines per treatment were collected from around the crown using pole clippers for the assessment of visible injury and elemental concentration analyses. The occurrence of chlorotic and necrotic tips over 0.5 mm long, stomatal chlorosis and other discolorations was assessed in 10 needles per tree from the middle part of the needles from the three youngest needle age classes. Needle length was measured with a ruler, and the whole needle area was estimated using a method described by Flower-Ellis and Olsson (1993).

TABLE 1

Cumulative amounts of sulfur (S), copper (Cu), and nickel (Ni) (mg m^{-2}) added to the experimental plots at the Kevo site during 1991–1996 (Neuvonen, S., unpublished).

	IC	pH3	CuNi			CuNi + pH3		
Year	S	S	S	Cu	Ni	S	Cu	Ni
1991	0	626	7	8	5	633	8	5
1992	103	3092	126	27	17	6115	27	17
1993	280	5621	318	45	28	5659	45	28
1994	347	6591	392	53	33	6636	53	33
1995	461	8622	519	69	43	8680	69	43
1996	587	10853	660	87	54	10926	87	54

Microscopy

Samples for anatomical observations were collected from five pines, different from those used for the elemental analyses, evaluation of visible injuries, and needle length and area measurements, to reduce destructive sampling from the same tree individuals. Ten fascicles from the middle section of the current and previous year's growth (C and C+1) were collected and treated as presented by Kukkola et al. (1997). Semithin sections of three to five needles per tree (about 2–3 μm) were stained with toluidine blue. Mesophyll cell damage was classified at the light microscopy level as presented by Bäck et al. (1994).

The point counting of the intercellular space, the cells in the various injury classes and the image analyzer measurements of the proportions of epidermis + hypodermis, mesophyll and transfusion layer from the total area were done on one to five trees and 5 to 15 needles per treatment, using current and previous year's needles (see Kukkola and Huttunen [1998] for method).

Thin sections for electron microscopy were made of two needles from each tree, using the same sample blocks as for semithin sections. Observations were made on three to five trees and four to five needles per treatment. Ultrathin sections stained with uranyl acetate and lead citrate were collected on copper grids and evaluated under a JEOL JEM 100 CX scanning transmission electron microscope. Hardening status was assessed by classifying (0%, 1–25 %, 26–50 %, 51–75 %, and 76–100 %) the seasonally typical endoplasmic reticulum (ER) forms of cytoplasm as presented by Sauter et al. (1996) and Jokela et al. (1998). The cells were divided into three classes according to ER appearance: (1) longitudinal ER, typical during the growing season, (2) vesicular and tubular ER, typical of hardened cells, and (3) ER structure not distinguishable. Other characteristics related to hardening (Soikkeli, 1980; Sauter et al., 1996), such as a decreased number of starch-containing chloroplasts (%), irregular shape of chloroplasts and elongated shape of mitochondria, were also classified. Injuries at the

TABLE 2

Element concentrations (mean with SD) in the two youngest needle age classes. Statistically significant $F_{(treatment\ df,\ error\ df)}$ *values are given for needle age and treatment.* F*-value for treatment is given separately for both needle age classes only in the cases where the treatment × needle age interaction was significant. Treatment means marked with* ***bold*** *differ significantly from IC (tested with LSD).*

	Needle age		Treatment					
Variable	Class	$F_{(1,16)}$	DC	IC	CuNi	pH3	CuNi + pH3	$F_{(4,16)}$
S ($\mu g\ g^{-1}$)	C	5.47*	1032.6 (104.4)	1146.1 (85.8)	1058.3 (163.7)	1132.3 (82.4)	1130.6 (134.0)	1.07 ns
	C+1		1019.5 (57.1)	1065.3 (73.1)	1014.1 (68.2)	1087.1 (137.9)	1115.0 (173.4)	
Cu ($\mu g\ g^{-1}$)	C	1.0 ns	3.7 (0.9)	4.3 (1.5)	5.9 (1.6)	6.0 (1.5)	6.1 (2.7)	1.8ns
	C+1		2.8 (0.4)	4.1 (1.5)	**7.7 (2.5)**	**7.2 (2.4)**	5.5 (2.6)	5.95**
Ni ($\mu g\ g^{-1}$)	C	12.51**	2.7 (0.3)	2.3 (0.2)	**3.5 (0.7)**	3.0 (0.8)	2.5 (0.5)	
	C+1		1.6 (0.3)	1.7 (0.2)	**3.5 (0.8)**	1.8 (0.4)	2.3 (1.3)	6.44**
Zn ($\mu g\ g^{-1}$)	C	10.7*	**40.9 (13.0)**	51.1 (7.6)	44.8 (7.6)	57.7 (5.7)	50.1 (6.7)	
	C+1		**48.8 (14.6)**	67.8 (13.1)	61.9 (11.7)	71.6 (10.2)	53.1 (4.7)	2.73°

Abbreviations: C, current year's needles; C+1, previous year's needles; DC, dry control; IC, irrigated control; pH3, acid rain; CuNi, metal treatment; CuNi+pH3, combination of metal and acid treatment; *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, ° = $P < 0.1$, ns = $P > 0.1$.

ultrastructural level were evaluated as described by Holopainen et al. (1992), and frost and acid rain-induced injuries were evaluated as described by Sutinen (1992) and Bäck (1994).

Element Analyses

The nickel (Ni), copper (Cu), sulfur (S), and zinc (Zn) concentrations were determined from unwashed current and previous year's pine needles using X-ray analyses (Siemens SRS AS X-ray fluorescence spectrometer). A Rh anode and the following crystals were used: Lif 100, PET, Ge and OVO 55. Both international and *ad hoc* standards were used for calibration purposes (see Rautio et al., 1998, for details).

Statistical Analyses

The element concentrations, the visible injuries of needles and the morphological data were analyzed using analysis of variance. The following classifying factors were used: block (five blocks), irrigation treatment (dry control, irrigated control, CuNi, pH3, and CuNi + pH3) and needle age (two needle age classes, except in the case of visible injuries, where three age classes were distinguished). Because needle age classes are not independent variables but can be considered as repeated measures, needle age class was considered as a within-subject factor.

Parameters indicative of (1) the proportions of ultrastructural symptoms in mesophyll cells (Table 4), and (2) the hardening status of chloroplasts, mitochondria, and cytoplasm and the proportion of starch-containing chloroplasts in mesophyll cells (Table 5) could not be measured from each sample because the preparation of thin sections for electron microscopy failed. This resulted in an unbalanced design with empty cells, and the sum of squares (SS) type IV was hence used to assess the statistical significance of these parameters. However, in the cases of all the other parameters, SS type III was used (Tabachnick and Fidell, 1996).

Because the block term was included in the above model only to decrease the margin of error, we report here *F*-values (and significances) for the irrigation treatment and needle age terms only. If the interaction between irrigation treatment and needle age was significant (indicating different responses to treatments by old vs. young needles), the effects of treatment (with the block effect) were tested separately for the different needle age classes. Pairwise comparisons between irrigated controls vs. other treatments were performed by using the LSD procedure (Saville, 1990).

Results

Six years of highly acidic input had no significant effect on forest soil acidity: average pH was 4.1 ± 0.3 (±SD) on the irrigated control plots and 4.0 ± 0.4 on the plots that received acid irrigation, which points to direct effects of metals and acid rain on the trees. The Ni and Cu contents differed significantly between C and C + 1 needles. While Ni content was higher in C needles in the case of Cu, this difference

TABLE 3

Proportion of visible injuries (%) in the three youngest needle age classes per tree (mean with SD). See Table 2 for abbreviations and details.

	Needle age		Treatment					
Variable	Class	$F_{(2,32)}$	DC	IC	CuNi	pH3	CuNi + pH3	$F_{(4,16)}$
Stomatal chlorosis	C		0	0	0	4 (5.5)	2 (2.7)	
	C+1	14.03***	10 (22.4)	21 (44.2)	14 (13.9)	18 (35.0)	26 (29.2)	0.76ns
	C+2		36 (29.0)	44 (34.2)	13 (16.0)	17 (20.0)	55 (45.7)	
Other discolourations	C		1 (2.2)	1(2.2)	0	0	1 (2.2)	
	C+1	5.03*	1 (2.2)	5 (5.0)	2 (4.5)	5 (8.7)	3 (2.7)	0.75ns
	C+2		10 (9.3)	1 (2.2)	2 (2.7)	3 (4.5)	7 (5.7)	
Tip chlorosis	C		0	0	0	0	0	
	C+1	1.86ns	0	2 (4.5)	0	0	0	0.82ns
	C+2		4 (9.0)	4 (9.0)	0	0	2 (4.5)	
Tip necrosis	C		2 (4.5)	0	0	0	0	
	C+1	3.74*	0	4 (5.5)	0	0	4 (5.5)	0.66ns
	C+2		0	1 (2.2)	6 (9.0)	4 (8.9)	10 (22.4)	

TABLE 4

Proportions (%) of ultrastructural symptoms in mesophyll cells (mean with SD) in the two youngest needle age classes. ER = endoplastmic reticulum. For other abbreviations and details, see Table 2.

	Needle age		Treatment					
Symptom	Class	$F_{(1,4)}$	DC	IC	CuNi	pH3	CuNi + pH3	$F_{(4,\#)}$
Chloroplasts								
Thylakoid swelling	C	2.94ns	56.2 (42.7)	16.7 (28.9)	38.9 (53.6)	26.7 (43.5)	33.3 (57.7)	0.39ns
	C+1		23.8 (18.0)	24.1 (35.4)	**39.4 (18.3)**	7.0 (12.2)	**34.8 (36.2)**	2.8°
Dark stroma	C	24.8**	0	0	0	20.0 (44.7)	0	
	C+1		7.0 (4.0)	7.0 (14.0)	0	1.8 (3.1)	7.2 (8.3)	0.62ns
Mitochondria								
Swelling	C	0.92ns	0	0	0	0	0	
	C+1		0	0	0	3.5 (6.1)	10.7 (21.5)	0.14ns
Cytoplasm								
Vacuolation	C	13.75*	50.0 (40.8)	50.0 (50.0)	88.9 (19.2)	50.0 (50.0)	72.2 (25.5)	
	C+1		7.7 (9.2)	21.8 (27.5)	64.0 (9.6)	92.2 (13.6)	73.4 (39.3)	1.15ns
Lipid accumulations	C	0.0ns	0	33.3 (57.7)	0	0	0	
	C+1		39.0 (21.4)	8.3 (14.4)	9.7 (8.7)	32.0 (38.7)	29.2 (34.4)	0.5ns
Dark accumulations	C	0.12ns	12.5 (25.0)	16.7 (28.9)	0	6.7 (14.9)	46.7 (50.3)	1.84ns
	C+1		10.6 (9.4)	6.7 (7.8)	**52.0 (36.1)**	14.0 (24.39)	18.4 (27.0)	1.94ns
ER disintegration	C	0.0ns	50.0 (0)	83.4 (23.5)	75.0 (35.4)	62.5 (47.9)	75.0 (35.4)	
	C+1		13.7 (9.2)	66.1 (47.2)	65.8 (8.0)	55.7 (39.3)	70.3 (39.5)	0.82ns
Plasmolysis	C	10.6*	12.5 (14.4)	11.1 (19.2)	0	10.0 (22.4)	31.1 (30.1)	
	C+1		23.3 (15.2)	34.8 (44.6)	2.8 (4.8)	3.5 (6.1)	34.5 (22.2)	0.23ns
Tonoplast breakdown	C	0.2ns	0	0	0	20.0 (44.7)	0	
	C+1		23.0 (11.4)	2.4 (4.1)	2.8 (4.8)	22.0 (25.6)	3.6 (7.2)	0.7ns

The error df varies within 13–17, except when the effect of treatment is tested separately for the different needle age classes df = 9.

varied between the different treatments. In the CuNi addition and acid treatments, which were significantly different from the irrigated control, Cu levels were higher in C + 1 needles, whereas in the other treatments Cu levels were slightly higher in C needles (Table 2). Zn content was higher in C + 1 needles, and even though Zn was not added into the irrigation water, plain irrigation caused Zn levels to rise more than in the dry controls, and pH3 treatment increased this difference even more (Table 2). CuNi treatment increased the foliar Ni concentrations significantly more than plain irrigation. The other nutrients did not differ between the treatments (Rautio, P., unpublished). Even though older needles usually had significantly more visible injuries than current needles, the treatments did not have a significant impact on these differences (Table 3).

In all treatments and in both needle age classes most cells had slight injury. The proportion of intercellular space varied from 20 to 34%. Needle length varied from 22 to 30 mm and total needle area from 721 to 1279 × 1000 µm^2. No statistical difference was observed between the treatments (data not shown).

Significantly increased ultrastructural symptoms, thylakoid swelling in the CuNi and CuNi + pH3 treatments, and dark accumulations in the CuNi treatment were observed when compared to the irrigated controls, (Table 4, Fig. 3). Trees exposed to CuNi and CuNi + pH3 had more swollen thylakoids in older needles, and CuNi-treated ones also had more dark accumulations than the irrigated controls (Table 4).

Needle cells were at the beginning of their hardening process in late September. Chloroplasts had moved from the proximity of cell walls to cell corners, and their shape was irregular. Compared to the irrigated controls (IC), cells collected from the trees in the dry control (DC) category and exposed to CuNi and CuNi + pH3 treatments had less elongated mitochondria (year classes pooled, Table 5). The proportion of starch-containing chloroplasts out of the total number of chloroplasts in mesophyll cells varied from 24 to 48%. Cytoplasm had vesicular and tubular instead of longitudinal ER (endoplasmic reticulum), and small lipid droplets were also observed (Table 5, Fig. 2A–B). In CuNi-treated needles, mesophyll cells occasionally had abundant membraneous structures in their cytoplasm. Large numbers of ribosomes were seen in cytoplasm and chloroplasts, and club-like chloroplasts were observed (Fig. 3a–b). However, in spite of the qualitative differences, none of these parameters (in Figs. 2, 3) differed significantly between the treatments.

Discussion

Metal treatments at the levels of Cu 8–19 and Ni 5–12 mg m^{-2} yr^{-1} over a period of 6 yr increased the concentrations of Cu in previous year's needles and Ni in both current year's and previous year's needles. It is also noteworthy that, in the pH3 treatment (with no CuNi addition), the concentration of Cu was higher than the control level in previous year's needles. This result may be due to the acid input into the soil, which may lead to increased Cu mobility. The slight trend in this direction seen in Zn would also point to increased metal mobility, since Zn was not given in any of these treatments. However, none of the element concentrations exceeded the limit of deficiency or toxicity. Low concentrations of Cu and Zn, for example, are required for enzymes. The element concentrations of healthy Scots pine current year's needles in southwestern Finland were Cu: 2.69–2.74 µg g^{-1} and Zn: 42–43 µg g^{-1} (Raitio, 1990). The needle Ni concentrations in this study did not differ from those measured in the current year's needles of Scots pines in the background area (Rautio et al., 1998).

We cannot, however, exclude the potentially harmful effects of metal addition on the nutrient balance of trees. There are other influential factors present in these regions, such as overgrazing by reindeer. Väre (1996), when studying the impacts of reindeer grazing and Cu and Ni deposition on forests in Northern Finland and on the Kola Peninsula (northwest Russia), observed that grazing decreased exchangeable nutrients by 30 to 60% in the organic layer. Further, grazing decreased soil moisture during the dry period. Väre (1996) also

TABLE 5

Hardening status of chloroplasts, mitochondria and cytoplasm and proportion of starch-containing chloroplasts in mesophyll cells. ER = endoplasmic reticulum. For other abbreviations and details, see Table 2.

	Needle age		Treatment					
Variable	Class	$F_{(1,4)}$	DC	IC	CuNi	pH3	CuNi + pH3	$F_{(4,\#)}$
Chloroplasts								
Irregular shape	C		40 (20.6)	18.1 (21.4)	62.0 (1.8)	62.3 (33.3)	46.4 (7.2)	
	C+1	32.1**	0	5.6 (9.6)	0	10.0 (22.4)	0	1.5ns
Starch	C		31.4 (12.2)	30.1 (21.9)	38.5 (5.3)	24.1 (20.5)	38.1 (9.8)	
	C+1	4.4ns	40.4 (4.5)	40.8 (9.9)	48.4 (6.0)	39.0 (15.1)	46.7 (19.0)	0.73ns
Mitochondria								
Elongated shape	C		**2.5 (5.0)**	22.5 (15.0)	30.0 (26.5)	**30.0 (26.5)**	**2.5 (5.0)**	
	C+1	2.9ns	**12.5 (14.4)**	83.3 (28.9)	16.7 (28.9)	**10.0 (13.7)**	**16.7 (28.9)**	5.22**
Cytoplasm								
Longitudinal ER	C		15.7 (21.0)	4.2 (8.35)	8.3 (14.4)	1.8 (3.1)	0	0.64ns
	C+1	0.28ns	12.5 (25.0)	0	11.1 (19.2)	20.0 (44.7)	16.7 (28.9)	
Tabular/vesicular ER	C		19.7 (23.3)	4.2 (8.35)	20.6 (4.2)	7.0 (12.2)	16.7 (33.4)	0.34ns
	C+1	0.9ns	25.0 (28.9)	33.3 (57.7)	22.2 (38.5)	30.0 (44.7)	27.8 (25.5)	

The error df varies between 13–17.

noticed that Cu and Ni cause reductions in soil microbial parameters similar to those caused by grazing, though more severe.

The differences in visible injuries between the treatments were not statistically significant, and stomatal chlorosis in current year's needles was only present in the pH3 and CuNi + pH3 treatments. Stomatal chlorosis has been observed to be related to air pollution in subarctic forests. According to Rautio et al. (1998b), visible damage was more abundant in older than in younger needles, and needle injuries were distinctly more abundant in the area within 50 km from Monchegorsk than in the other areas. Some stomatal chlorosis may be caused by acid deposition and the increased solubility of copper and nickel resulting from the increased acidity of the water film or droplets on the needle surface, which enhances the erosion of epicuticular waxes (Milne et al., 1990). Acid irrigation has been reported to cause an increased incidence of poorly developed epistomatal waxes and deformed stomata (Turunen and Huttunen, 1991), which may also explain the poorer condition of current year's needles in the pH3 and CuNi + pH3 treatments compared to the other treatments.

There were no additional differences in needle morphology between the treatments after 6 yr of acid and/or metal addition compared with the observations made at 4 yr (Kukkola and Huttunen, 1998), although pines did have shorter needles in the metal treatment than in the pH3 treatment (Turunen, 1996). Hence, the additional 4262 mg S m^{-2} in the acid treatment or the 34 mg of Cu and 21 mg of Ni m^{-2} given in the CuNi treatment during the 2 yr after the study by Kukkola and Huttunen (1998) (cf. Table 1) do not seem to have exceeded the tolerance level of this system.

Thylakoid swelling and dark accumulations were the most pronounced symptoms in CuNi- and CuNi + pH3-treated previous year's needles, which points to metal-induced disintegration in chloroplast structure and metal accumulation within cells. Symptoms related to frost injury (Ziegler and Kandler, 1980) were seen in

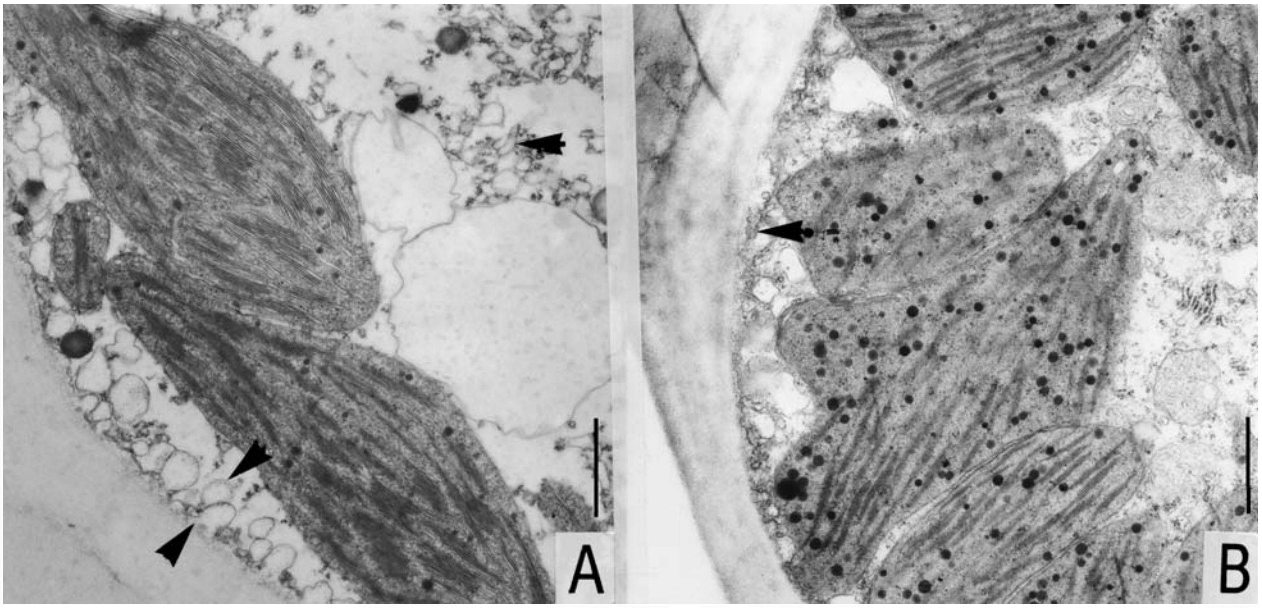

FIGURE 2. Transmission electron micrographs of mesophyll cells of previous year's pine needles at different stages of hardening. (A) Irrigated control (IC): hardened cytoplasm with tubular ER (arrow) and vesicular ER (between arrows near the plasma membrane); small lipid droplets. (B) Dry control (DC): the hardening process has just begun; longitudinal ER left in cytoplasm (arrow). Bar = 1µm.

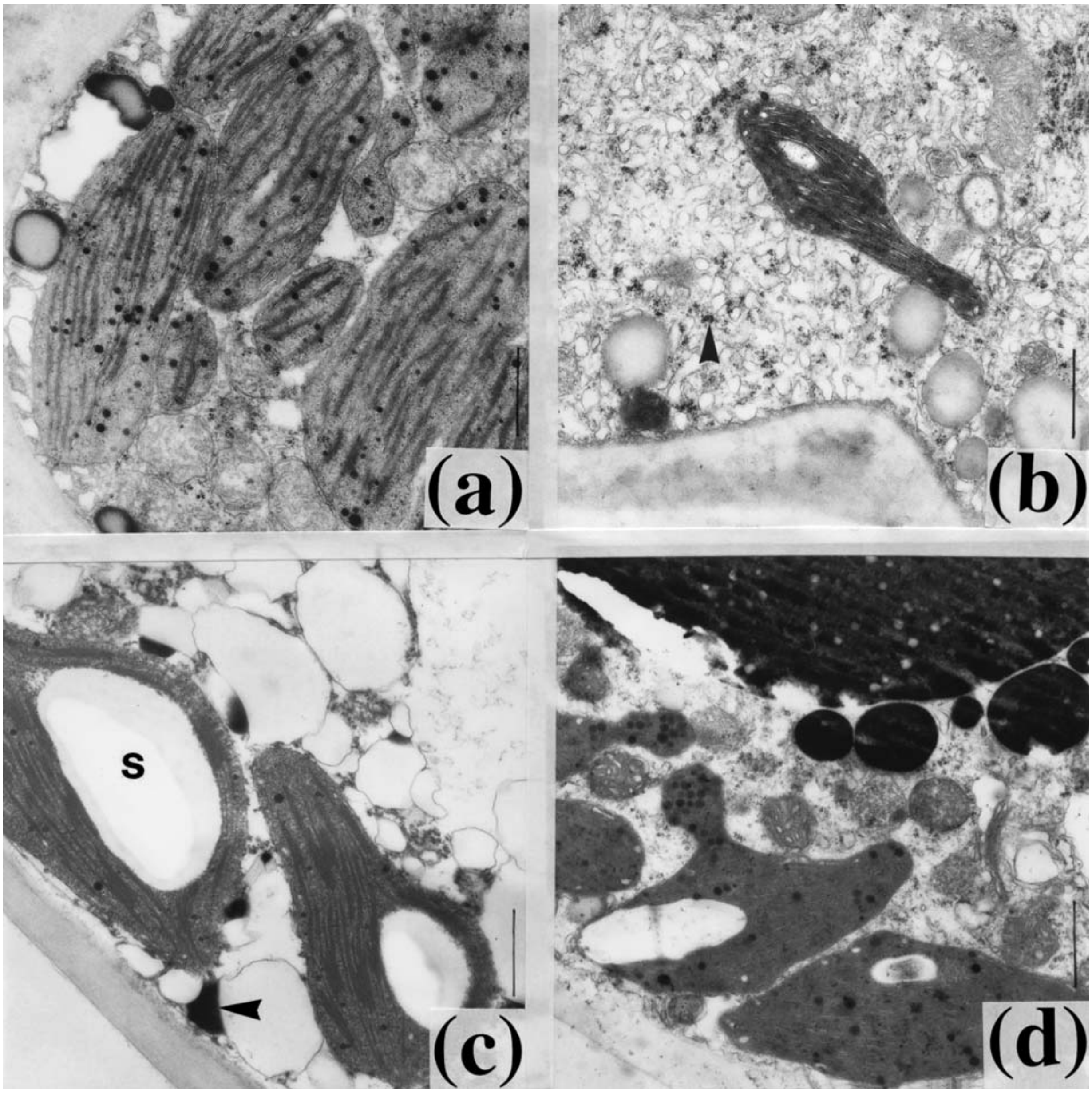

*FIGURE 3. Transmission electron micrographs of damage symptoms observed in previous year's mesophyll cells of pine needles. (a) Dry control (DC): intact cell. (b) Cu + Ni treatment: Dense cytoplasm with a large number of ribosomes in both cytoplasm and chloroplasts (arrow). Notice the club-like shrunken chloroplast. Thylakoid membranes of chloroplasts appear to be swollen. (c) pH3 treatment: Vacuolation of cytoplasm. Note the dark accumulations in cytoplasm (arrow). (d) pH3 treatment: Protrusions in chloroplasts. Bar = 1*µ*m, s = starch grain.*

previous year's needles in all the treatments (disintegrated ER also in C needles). Tonoplast breakdown was seen in previous year's needles in all treatments, pointing further to severe frost injury (Ziegler and Kandler, 1980; Reinikainen and Huttunen, 1989). Both vacuolation and swelling of thylakoids seem to be related to needle development and reversible to some extent (Anttonen et al., 1996; Jokela et al., 1998), because they were seen abundantly in current year's needles that had not yet overwintered.

The increase of cytoplasmic membranes observed in the CuNi treatment may be related to cation excess. At sublethal concentrations, the lipids and proteins of these membranes may tie up excess quantities of intracellular cations, as suggested earlier by Visviki and Rachlin (1994) in the case of copper-exposed *Chlamydomonas bullosa* cells and by Rachlin et al. (1982) in the case of copper- and nickel-exposed *Plectonema boryanum* cells, though this mechanism has not been studied in higher plants.

The most pronounced type of injury in the mesophyll cells of Scots pine needles was complete vacuolation of the cytoplasm. At the ultrastructural level, Cu and Ni seemed to cause vacuolation, dark accumulations, thylakoid swelling, and plasmolysis in chloroplasts in the CuNi and/or CuNi + pH3 treatments. These injuries were not distinguishable from frost injuries in a preliminary study at Kevo (Kukkola and Huttunen, 1998). But in this study, the treatment-related changes consisted of thylakoid swelling and dark accumulations in older needles. This type of injury is clearly affected by acid rain, and, even here, we observed a clear trend for cytoplasm vacuolation in previous year's needles in the pH3 and CuNi + pH3 treatments compared to the irrigated control. Frost injuries, e.g., thinned cytoplasm, and dark-stained material deposits in the margins of cytoplasm or in chloroplasts were abundant. However, these were not related to the treatments, because the progression of the winter hardening process did not differ between the treatments, except in the case of the elongated shape of mitochondria.

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