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## Antioxidative Systems in Spruce Clones Grown at High Altitudes

By

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**Key words:** Ascorbate, genetic variation, glutathione, glutathione reductase, ozone, peroxidase, superoxide dismutase.

### Summary

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Antioxidative systems were studied in current-year needles of 15 different clones of four-year-old spruce trees (*Picea abies* (L.) Karst.) grown at three altitudes (800 m, 1150 m and 1750 m above sea level) and compared with those in mature spruce trees after life-time growth at these sites. In both mature spruce trees and clones antioxidative systems increased and chlorophyll decreased with increasing elevation. In general, the increase in antioxidative systems was less pronounced and chlorophyll reductions were higher in clones than in mature spruce trees. Ozone-tolerant clones did not display higher antioxidant contents and superoxide dismutase or guaiacol peroxidase activities than ozone sensitive clones. The maintenance of enhanced chlorophyll b contents in tolerant clones with increasing elevation suggests that stress compensation was associated with other factors than the antioxidants analysed.

### Introduction

At high altitude, forests fulfill important functions such as protection against soil erosion, landslides, and avalanches. Therefore, the health, vitality and stability of these forest ecosystems is of particular significance. However, in many forests grown at high altitude up to 50 or 60 % of the trees show slight or even

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severe symptoms of injury such as yellowing and/or loss of needles (RENNENBERG & al. 1997). In the Calcareous Alps, Norway spruce is the dominant tree species representing about 70% of all forest trees. At Mt. Wank (Bavaria, Germany, peak 1785 m above sea level) symptoms of injury increased with increasing elevation and showed a strong progression in time (RENNENBERG & al. 1997). One of the reasons for the poor health of spruce in this area are nutrient deficiencies or limited nutrient availability (POLLE & al. 1992). However, unbalanced nutrition can not explain the increasing degree of injury observed with increasing altitude.

At high altitude, trees are exposed to unfavorable climatic conditions such as low temperatures, high irradiance, enhanced UV-B radiation and elevated ozone (RENNENBERG & al. 1997, STOCKWELL & al. 1997). On top of Mt Wank the mean temperature (12 °C) in the growth phase is about 5 °C lower than at valley level, whereas mean ozone concentrations are about 2-fold higher. These adverse factors may act in plants through the generation of reactive oxygen species, but can be combated by antioxidative systems (FOYER & al. 1994, POLLE 1997). Autochthonous spruce trees grown at high altitudes at Mt. Wank at 1700 m a.s.l. generally show increased levels of antioxidants and protective enzymes in their needles as compared with valley level (735 m a.s.l.) (POLLE & RENNENBERG 1992). Similar observation have also been reported for spruce trees and other species grown in other mountaneous field sites (STREB & al. 1997, WILDI & LÜTZ 1996, GRILL & al. 1988). However, the activity of superoxide dismutase was not increased in needles from trees grown at high altitudes close to the tree line (POLLE & RENNENBERG 1992, MADAMANCHI & al. 1991). It is, therefore, unclear whether the observed changes in antioxidants provide sufficient protection to prevent premature needle loss.

The major questions of our study were: (1) do antioxidative systems fail in a stressfull climate, thereby, putting the trees at risk for oxidative injury and (2) do environmental factors or inherited features (genotype) play a major role in predisposing trees for injury at Mt. Wank. Since ozone is discussed as one of the factors contributing to the injury of forest trees, clonal spruce trees with varying degree of ozone sensitivity (SCHOLZ & VENNE 1989) were planted into natural soil at three altitudes at Mt. Wank. The field sites were located close to valley level (800 m a.s.l.), at intermediate elevation (1150 m a.s.l.) and close to the tree line (1700 m a.s.l.). The present study reports on antioxidants and tree performance after one year of exposure.

## Materials and Methods

### Plant material

Three-year-old clonal spruce trees (*Picea abies* (L.) Karst.) were obtained from the Bundesforschungsanstalt für Forst- und Holzwirtschaft (Institut für Forstgenetik and Forstpflanzenzüchtung, Großhansdorf, Germany). The clones originated from provenances of the Hercynic-Carpathian distribution area from 650 to 1130 m a.s.l. Among the 15 clones used in the present study, 8 clones had previously been tested for ozone tolerance by exposing them to high acute ozone levels until some clones displayed serious symptoms of needle loss, whereas others showed no visible signs of injury (SCHOLZ & VENNE 1989). The clones were ranked according to

injury, high R-values indicating ozone sensitivity and low R-values indicating ozone tolerance. At Mt. Wank, three experimental plots were established at the south-west facing slope at altitudes of 800 m, 1150 m, and 1750 m a.s.l. Each plot was subdivided into four repetitions in which four individuals of each clone were planted into the natural forest soil at a distance of 2 m in a row as shown in figure 1. Thus, each plot contained 16 individuals of each clone. After one year of growth at Mt Wank current year needles from the 2<sup>nd</sup> whorl were harvested in September. At each site, the needles of each clone were pooled and frozen in liquid nitrogen for biochemical analyses. In addition to clonal material, current-year needles of 16 mature trees were harvested on each site and pooled as for the clones.

#### Biochemical analysis

Frozen needles were powdered under liquid nitrogen and extracted for enzyme analysis in 100 mM phosphate buffer pH 7.8 containing 1 % Triton X-100 and 4 % insoluble polyvinylpyrrolidone or for antioxidant analysis in 0.1 N HCl containing 4 % insoluble polyvinylpyrrolidone (POLLE & al. 1992). The activities of SOD, POD, and GR were determined spectrophotometrically after standard protocols (FOYER & HALLIWELL 1976, POLLE & al. 1990). Ascorbate and glutathione were determined by the HPLC methods described elsewhere (POLLE & al. 1990, SCHUPP & RENNENBERG 1988). Protein was determined with bicinchoninic acid after gel filtration over Sephadex G-25 (Pharmacia, Freiburg, Germany). The needles were oven dried for 72 hours at 80°C to obtain dry matter. The pigments were determined in 80 % acetone using the extinction coefficients reported by LICHTENTHALER & WELLBURN 1983. Each sample was analysed in triplicate.

#### Statistical analysis

Data in figures or tables indicate mean ( $\pm$ SD). The data were analysed by ANOVA employing a multiple range test (LSD) to detect differences significant at  $P \leq 0.05$  (Statgraphics, St. Louis, Mo). The Friedman test was used for rank analysis.

### Abbreviations

Asl = above sea level, GR = glutathione reductase, Mt = mount, P = probability, POD = peroxidase, SD = standard deviation, SOD = superoxide dismutase,

### Results and Discussion

One year after the clones had been planted, mortality amounted only 2 out of 720 plants. The relative dry matter of spruce needles amounted  $41 \pm 2$  % regardless of whether young clonal or mature trees were studied (Table 1). As observed previously at Mt Wank and in other alpine habitats (POLLE & al. 1992, STREB & al. 1997), the chlorophyll content of the foliage generally decreased with increasing elevation, whereas the carotenoids were little affected (Table 1). Needles from clonal spruce tree displayed 40% lower chlorophyll contents than those of mature trees, but carotenoid contents were similar in both needles from clones and mature trees (Table 1). Because of the strong reduction in chlorophyll in clonal trees, the carotenoid-to-chlorophyll-ratio was increased in these plants, especially at high altitude (1.6-fold) and higher than in needles of mature trees (Table 1). This observation suggests a relative increase in photoprotection in the clones as





compared with autochthonous spruce trees. The protein contents of needles from mature trees were similar to those in clonal trees with the exception of high protein contents in mature trees at the middle elevation (Table 1). In a previous study with autochthonous spruce trees at Mt. Wank, the protein contents of needles were comparable to those found here ranging from 43.7 to 45.7 mg g<sup>-1</sup> dry mass at the three altitudes (POLLE & al. 1992).

Table 1. Pigment and protein contents per gram of dry mass in current year needles of clones and mature spruce trees at three altitudes at Mt. Wank. The needles were harvested in September 1992 and analysed as described under Materials and Methods. Data indicate means (n = 16 for clonal trees, n = 3 for mature trees,  $\pm$  SD). DM/FM = dry mass-to-fresh mass ratio.

Plant	Altitude (m asl)	Chl a (mg)	Chl b (mg)	Car (mg)	Chla/ Chlb	Car/Chl	Protein (mg)	DM/ FM (%)
Clone	800	8.9 $\pm$ 2.1	2.1 $\pm$ 0.4	3.4 $\pm$ 0.5	4.29	0.307	52.3 $\pm$ 11.7	42.6
Old	800	12.9 $\pm$ 0.5	3.1 $\pm$ 0.1	3.5 $\pm$ 0.1	4.21	0.219	52.1 $\pm$ 2.1	41.8
Clone	1150	10.1 $\pm$ 2.4	2.4 $\pm$ 0.6	3.7 $\pm$ 0.6	4.19	0.295	52.9 $\pm$ 11.7	40.8
Old	1150	11.9 $\pm$ 0.9	2.8 $\pm$ 0.2	3.3 $\pm$ 0.2	4.22	0.224	71.4 $\pm$ 3.2	37.2
Clone	1750	5.2 $\pm$ 1.0	1.2 $\pm$ 0.2	3.1 $\pm$ 0.6	4.41	0.487	42.1 $\pm$ 9.5	41.2
Old	1750	9.4 $\pm$ 0.2	2.0 $\pm$ 0.1	3.4 $\pm$ 0.1	4.63	0.299	49.9 $\pm$ 2.9	42.7

At Mt. Wank antioxidants in needles of mature spruce trees were found to fluctuate seasonally with generally higher contents at the tree line than at valley level (POLLE & RENNENBERG 1992). In the present study mature trees were investigated only in few replicates. However, the results were similar to those observed previously. In needles of clonal trees, the foliar concentrations of ascorbate and glutathione did not increase with increasing elevation, whereas those in needles from mature trees did (Fig. 2A, B).

Moreover, the activities of SOD, POD, and GR were generally higher in mature than in clonal trees supporting the idea that stress compensation may be less sufficient than in autochthonous trees (Fig. 3). The activities of antioxidant enzymes in needles of mature trees did not respond consistently to altitudinal stress; e.g., SOD did not increase with increasing altitude as one might have expected (Fig. 3A). However, this has also been observed previously at Mt. Wank (POLLE & RENNENBERG 1992). More recent data suggest that the regeneration of antioxidants is limiting at high altitude (POLLE & al. 1999). From the present data it appears that clonal spruce trees experienced higher stress than mature trees because the physiological stress acclimation of clonal spruce trees was lower than that of mature trees after life-time exposure. This difference may have been caused by environmental constraints or by inherited features.

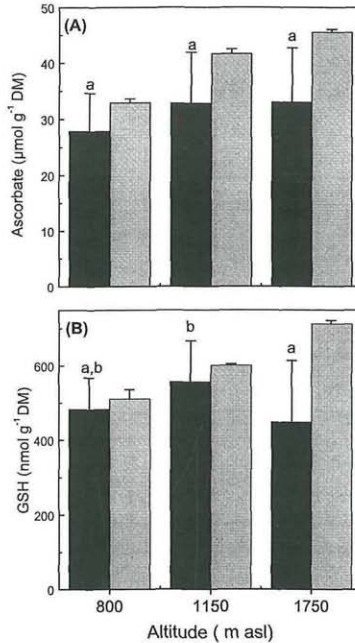


Fig. 2. Ascorbate and glutathione in current-year needles of young clones and mature spruce trees at three altitudes at Mt. Wank. The needles were harvested in September 1992 and were analysed as described under Materials and Methods. Data indicate means  $\pm$  SD ( $n = 16$  for clonal trees,  $n = 3$  for mature trees). Different letters indicate significant differences at  $P < 0.05$  for clonal trees. Mature trees were not included in the statistical analysis because of the small number of independent replicates.

In order to investigate whether the magnitude of the antioxidative system was related to ozone tolerance, the clonal spruce trees were ranked according to  $R$ -values representing increasing ozone-sensitivity (Table 2). Trees with  $R$ -values  $< 1.5$  were relatively ozone resistant as compared with those displaying  $R$ -values  $> 2$ . When the antioxidants of the two groups, resistant and sensitive trees, were compared by rank analysis, neither ascorbate, nor glutathione, superoxide dismutase or guaiacol peroxidase displayed significant effects. There is also evidence from other studies that none of these parameters is affected in spruce by exposure to moderate, relatively realistic ozone doses (POLLE & RENNINGER 1991, BAUMBUSCH & al. 1998, KRONFELD & al. 1998). However, we found that glutathione reductase activity was about 50% higher in ozone-sensitive than in ozone-tolerant clones (Table 2). This observation suggests that ozone-tolerant clones have an increased need for detoxification resulting in an increased turn-over rather than increased contents of antioxidants. The ozone sensitive clones contained elevated protein concentrations (+20%), but less chlorophyll b (-15%, Table 2).

Table 2. Protein, pigments and antioxidants in clonal spruce trees ranked after the degree of ozone resistance (R). Data are means (n = 3) expressed per gram of dry matter.

CLONE	R	Altitude (m)	Protein (mg)	Chl a ( $\mu\text{g}$ )	Chl b ( $\mu\text{g}$ )	Car ( $\mu\text{g}$ )	Asc ( $\mu\text{mol}$ )	GSH (nmol)	GR (nkat)	POD ( $\mu\text{kat}$ )	SOD (units)
C 4/4/10	0.62	800	42.07	1035	255	365	32.28	444	12.82	9.09	2195
C 4/4/10	0.62	1150	51.75	1067	261	379	39.43	478	6.74	6.40	1805
C 4/4/10	0.62	1750	36.11	590	155	347	35.43	356	17.13	4.62	1800
C 4/4/4	1.02	800	33.00	645	148	291	32.89	417	8.53	4.63	1341
C 4/4/4	1.02	1150	38.92	646	151	283	30.35	499	12.28	7.45	2239
C 4/4/4	1.02	1750	40.71	718	159	419	35.94	478	12.70	8.91	1958
C 5/1/16	1.06	800	56.89	796	208	301	35.97	481	23.35	2.10	1614
C 5/1/16	1.06	1150	53.80	1161	283	379	34.07	577	16.30	6.55	2329
C 5/1/16	1.06	1750	44.73	452	110	248	31.93	434	26.28	5.13	2164
C 4/4/18	1.36	800	48.14	1151	259	432	22.20		16.80	2.83	2791
C 4/4/18	1.36	1150	43.69	1030	252	391	23.54		10.66	4.01	2344
C 4/4/18	1.36	1750	46.13	569	112	346	21.80	536	11.01	4.86	1855
C 4/1/9	2.18	800	58.36	612	143	280	29.00	435	33.73	1.10	1568
C 4/1/9	2.18	1150	42.89	683	165	284	28.22	484	32.77	2.78	1830
C 4/1/9	2.18	1750	68.77	670	145	393	28.32	765	41.76	4.89	1810
C 4/4/13	2.47	800	50.46	706	179	291	34.03	361	11.87	8.73	2291
C 4/4/13	2.47	1150	43.89	893	215	334	21.89	622	12.80	7.00	1826
C 4/4/13	2.47	1750	55.53	468	110	268	19.62	267	13.71	10.60	1929
C 4/4/8	3.11	800	37.99	851	207	357	35.30	498	14.14	7.50	2269
C 4/4/8	3.11	1150	58.42	786	190	333	40.42	522	13.29	6.02	2233
C 4/4/8	3.11	1750	39.98	510	124	315	38.58	363	14.00	4.41	1146
C 6/3/10	3.95	800	79.73	885	181	384	13.12		30.07	4.99	2018
C 6/3/10	3.95	1150	60.82	1138	256	472	28.93	353	11.76	8.70	2977
C 6/3/10	3.95	1750	37.99	481	86	326	19.06		27.42	8.52	2124
P			0.083	0.564	0.021	1.0000	0.564	0.527	0.083	0.564	0.536

The relative reduction in chlorophyll b may point to injury at the level of photosystem II. It is now generally accepted that ozone does not directly injure the chloroplasts but may act through the production of secondary oxidising components (POLLE & PELL 1999). However, it is still obscure, how such reactions can cause specific responses at particular sites, e.g. in photosystems II or rubisco.

## Conclusions

The present data show that antioxidative systems in clonal spruce trees after one year of growth at high altitudes are less enhanced than those of mature

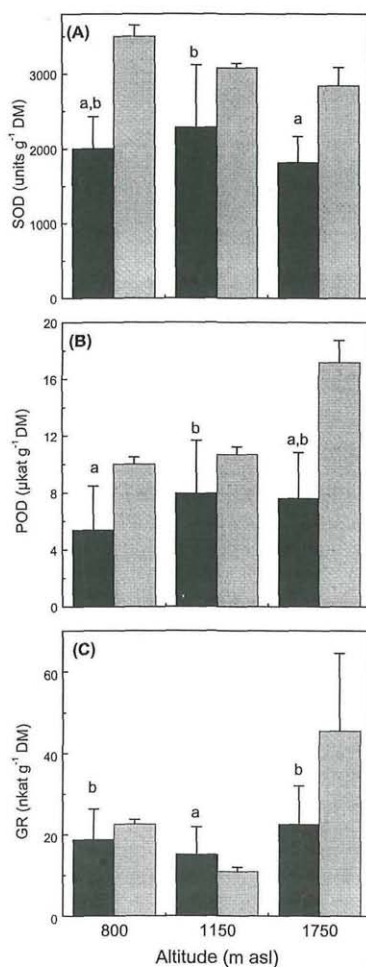


Fig. 3. Superoxide dismutase (A), peroxidase (B) and glutathione reductase (C) activities in current-year needles of clones and mature spruce trees at three altitudes at Mt. Wank. The needles were harvested in September 1992 and analysed as described under Materials and Methods. Data indicate means  $\pm$  SD ( $n = 16$  for clonal trees,  $n = 3$  for mature trees). Different letters indicate significant differences at  $P < 0.05$  for clonal trees. Mature trees were not included in the statistical analysis because of the small number of independent replicates.

trees after life-time exposure to these conditions. Apparently, the physiological acclimation process is slow or the clones might be unable to maintain increased antioxidants necessary to cope with altitudinal stress. Under ambient conditions the feature „ozone-tolerance“ previously determined by exposure to acute, toxic ozone



doses did not correlate with increased activities of protective enzymes or antioxidant levels. By contrast, ozone-sensitive clone displayed higher glutathione reductase activities than ozone-tolerant clones. The present results mark the beginning of a long-term experiment designed to address genetic x environmental interactions of spruce trees.

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