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Do Photoprotective Pigments and Antioxidants in Needles of *Pinus sylvestris* Relate to High N or Water Availability at Field Plots in a Dry Year?

By

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Summary

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Photoprotective pigments and antioxidants are widely used as stress markers in trees. We investigated these compounds in needles of mature *Pinus sylvestris* growing at three field plots (coded GR, KA, and LI) in Brandenburg, Germany, in a drought year (1999). The plots differ with respect to water availability and former exposure to high atmospheric N input. GR has lower water availability than KA and LI, and LI was exposed to very high atmospheric N input until 1990. Even 9 years later, LI needles still had higher needle N concentrations. Chlorophyll concentrations were lowest and carotenoid and tocopherol concentrations per mg chlorophyll were highest at GR, but similar between KA and LI. The epoxidation state of the xanthophylls was lower at GR and LI compared to KA. Ascorbate and glutathione concentrations and redox states were similar across the plots. We conclude that in this dry year needle photoprotective pigments and antioxidants were more related to low water availability (at GR) than to high N status (at LI).

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Introduction

Antioxidants and photoprotective pigments are widely used as markers of (photo-) oxidative stress in trees (TAUSZ & al. 2003). These substances protect plant cells from the deleterious effects of reactive oxygen species (ROS). ROS are formed at high rates in plant cells exposed to environmental stress (ELSTNER & OßWALD 1994). The major low molecular antioxidants in most leaves are ascorbate (SMIRNOFF & WHEELER 2000), glutathione (TAUSZ 2001), tocopherol (MUNNÉ-BOSCH & ALEGRE 2002), and carotenoids (DEMMIG-ADAMS 2003). Carotenoids are also involved in the harmless dissipation of absorbed light energy as heat (DEMMIG-ADAMS 2003).

In conifer needles, these substances respond to seasonal fluctuations (TAUSZ & al. 2002) and natural stresses, such as drought (TAUSZ & al. 2001), low temperatures, or high altitudes (POLLE & RENNENBERG 1992). Environmental pollutants such as ozone (KRONFUß & al. 1998) can also affect antioxidants and/or pigments. Visible pollution injury is preceded by changes in antioxidants and pigments (TAUSZ & al. 1999).

Forest trees currently have to face new challenges brought about by climate change. During the last decade, average temperatures increased and summer droughts were more frequent and severe in many forested regions in Europe. Drought will put a high demand on antioxidative defence systems of the needles, and in an Austrian mountain forest it has been shown that site factors indicative of the water supply of trees were related to antioxidative responses (WONISCH & al. 1999).

High N supply can lead to increased chlorophyll concentrations in pine needles (e. g. FIFE & NAMBIAR 1997) which may enhance the light capture capacity in the photosynthetic membranes. An excess of absorbed energy that is not consumed by assimilation brings about the stress-related formation of ROS (ELSTNER & OßWALD 1994). Excess N supply may therefore further destabilise the relation between light capture and energy consumption. High atmospheric N input occurs, for example, in the vicinity of livestock farms, which emit N in form of ammonia. Efforts to reduce such N emissions have been successful in the nineties and lead to the ongoing recovery of formerly damaged forest plots.

The potential effect of interactions of drought and former N damage on antioxidative and photoprotective compounds in needles of field grown trees has not been investigated. In the present study, we sampled three mature *Pinus sylvestris* plots that differed in their water availability and their N-status. This field study is embedded in a large-scale multidisciplinary program ("monitoring on permanent plots with biomarkers", WIENHAUS & al. 2002). Sampling was conducted in the exceptionally dry year 1999. We addressed the questions whether (1) the low-molecular antioxidants ascorbate, glutathione, and tocopherol as well as chloroplast pigments in pine needles vary among plots that have been exposed to different N input for long times, or (2) whether these substances vary in relation to the water availability at sites.

Table 1. Study plots near Eberswalde in Brandenburg, Germany. More details in WIENHAUS & al. 2002.

	Plot Groß Schönebeck (GR)	Kahlenberg (KA)	Lichterfelde (LI)
Coordinates	52° 58' 30" N 13° 38' 40" E	52° 49' 45" N 13° 53' 12" E	52° 49' 12" N 13° 48' 49" E
Elevation [m]	40	50	40
Mean annual precipitation [mm]	585	561	561
Mean annual temperature [°C]	8.3	8.2	8.2
Mean July temperature	17.5	17.5	17.5
Soil type	podzol	sandy brown soil	podzolic sandy brown soil
Potential natural vegetation (according to HOFMANN 1994)	*Melampyro-Fagetum sylvaticae*	*Majanthemo-Fagetum sylvaticae*	*Agrostio-Quercetum robori-petraeae*
Actual vegetation (according to HOFMANN 1994)	*Myrtillo-Cultopinetum sylvestris*	*Rubo-Avenello-Cultopinetum sylvestris*	*Calamagrostio-Cultopinetum sylvestris*
Immissions	moderate until 1990 low since 1993	moderate until 1990 low since 1993	very high N until 1990 low since 1993

Material and Methods

Sampling plots: Three different plots (coded GR, KA, and LI) were selected within 30 km of Eberswalde, Germany, to represent the range of *Pinus sylvestris* forest types in this area (Table 1). The vegetation period 1999 was clearly warmer (mean temperature by 2.1 higher than the long-term average in July, and by 3.2 degrees higher in September), and drier (mean precipitation in the vegetation period from April to September was about 50 to 70 mm below average, and before sampling there were 14-16 days without any precipitation) than the long-term average on these sites. Water availability among sites varied in the order LI (highest water availability) > KA > GR (lowest water availability), and was very low during the months prior to sampling (WIENHAUS & al. 2002, LÖFFLER 2004). LI was exposed to very high atmospheric N input from an adjacent hog feeding plant until 1990, when the plant was closed. While atmospheric input of SO_2 and NO_x were low due to the strong reduction of emissions since 1990, O_3 concentrations during 1999 exceeded thresholds for daily averages of 65 mg m^{-3} at 86 days.

Sample trees: Ten *Pinus sylvestris* L. trees were sampled at each of the three plots. Trees were dominants or co-dominants of the highest vitality group. Plot GR was sampled on September 14, plot KA on September 16, and plot LI on September 18, 1999. Sampling was done on clear, sunny days and lasted from 9:30 to 16:00 Middle European Summer Time at each plot. Climbers pruned branches from sun-exposed (southward) branches from the upper third of the canopy. Fascicles of the current and the previous season's age class (needle age 0 and 1) were removed separately and immersed in liquid N_2 within seconds. Fascicles were lyophilised. After removing the fascicle base (about 1 cm) the needles were ground in a dismembrator (Braun, Germany). The needle powder was stored frozen in humidity proof plastic vials before it was subjected to biochemical analysis.

The N concentration in the needles was determined by dry combustion using elemental analyser LECO CNS 2000 (BMELF 1994).

Optimum quantum efficiency of photosystem II (Fv/Fm) was measured by chlorophyll fluorescence analysis according to MAXWELL & JOHNSON 2000.

Biochemical analysis: Pigments, tocopherols, ascorbic acid, and glutathione were measured as recommended in TAUSZ & al. 2003. Mainly thylakoid-based compounds (carotenoids, tocopherol) were expressed per mg chlorophyll to relate their protective capacity directly to the potential energy absorption.

Statistics: Statistical evaluations were completed using Statistica (StatSoft, USA, 1994) software package. A two-way ANOVA with plot (GR, KA, LI) and needle age class (0, 1) as independent fixed factors was run. ANOVA assumptions were checked by Levene's test for homogeneity of variance, by visually plotting means against variances to assure that they were not correlated or dominated by outliers, and by visually checking normal and random distribution of the residuals. Logarithmic transformations of data removed significant inhomogeneities of variances where such existed. Fisher's least-squared differences evaluated post-hoc differences between groups.

Results

As shown in Table 2, the effects of plot and/or needle age were significant for some variables, whereas a significant interaction plot x needle age was not found.

Table 2. Two-way ANOVA results for the investigated variables. n=10 in each group. dw leaf dry weight, Chl chlorophyll. V+A+Z = violaxanthin + antheraxanthin + zeaxanthin. EPS = epoxidation state of V+A+Z.

	P-values of ANOVA effects		
	Needle age	Plot	Plot x Needle age
Total chlorophyll [mg g^{-1} dw]	0.005	0.036	0.803
Chlorophyll a [mg g^{-1} dw]	0.024	0.082	0.776
Chlorphyll b [mg g^{-1} dw]	<0.001	0.004	0.840
N [mg g^{-1} dw]	0.159	0.006	0.744
V+A+Z [mg g^{-1} total Chl]	0.096	0.005	0.413
Neoxanthin [mg g^{-1} total Chl]	<0.001	0.125	0.475
Lutein [mg g^{-1} total Chl]	<0.001	0.142	0.726
α-Carotene [mg g^{-1} total Chl]	0.036	0.001	0.369
β-Carotene [mg g^{-1} total Chl]	0.067	0.003	0.813
α-Tocopherol [mg g^{-1} total Chl]	<0.001	0.003	0.693
EPS [%]	0.042	0.003	0.566
Total ascorbate [mg g^{-1} dw]	0.001	0.476	0.534
Dehydroascorbate [%]	0.466	0.125	0.230
Total glutathione [nmol g^{-1} dw]	0.401	0.335	0.569
Oxidised glutathione [%]	0.003	0.476	0.640

Chlorophyll b and total chlorophyll concentrations were significantly different among plots (Table 2) with needles from plot GR showing significantly lower concentrations than those from plot KA (Fig. 1, Table 3). Irrespective of plot, 1-year-old needles had higher chlorophyll concentrations than current year's needles (Table 2, 3, Fig. 1).

Needle N concentrations were significantly higher at plot LI compared to GR and KA (Table 2, 3). Chlorophyll fluorescence analysis revealed no significant differences in Fv/Fm (maximum efficiency of photosystem II) among plots or needle age classes and all mean values were around 0.80 (data not shown).

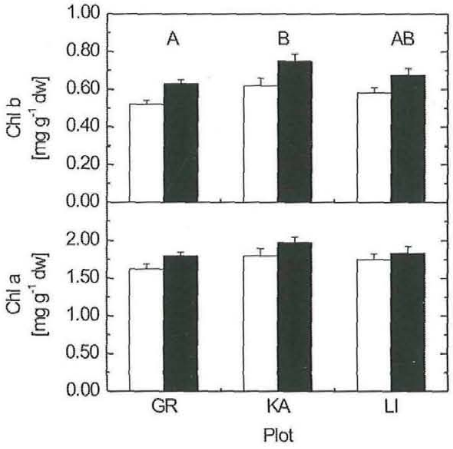

Fig. 1. Chlorophyll concentrations in current (white columns) and 1-year-old (black columns) needles of *Pinus sylvestris* growing at field plots in Brandenburg, Germany. Chl chlorophyll, dw needle dry weight. Means and standard errors of n=10 trees each. Plots significantly different from each other have no letter in common.

The concentrations of the defence systems mainly located in the thylakoids (carotenoids and tocopherol) were expressed per unit chlorophyll to relate them directly to the light capture potential. The concentrations of neoxanthin, lutein, α-carotene, and α-tocopherol were significantly different among needle age classes (Table 2). One-year-old needles had higher concentrations of those compounds per unit chlorophyll than current year's ones (Table 3, Fig. 2). The concentrations of the xanthophyll cycle pigments together (violaxanthin, V, antheraxanthin, A, and zeaxanthin, Z, V+A+Z), α-carotene, β-carotene, and α-tocopherol were significantly different among the plots (Table 2). V+A+Z and β-carotene concentrations were higher in needles from plot GR than in those from the plots KA and LI (Fig. 2, Table 3). α-Carotene concentrations were lower in needles from plot LI than those from the plots GR and KA (Table 3). α-Tocopherol concentrations were significantly higher at plot GR compared to KA with the plot LI as an intermediate (Fig. 2). Neoxanthin and lutein concentrations were similar

Table 3. Total chlorophyll (chlorophyll a+b), N concentrations and carotenoid concentrations per unit chlorophyll in *Pinus sylvestris* needles from plots exposed to different N input and summer drought. Means (standard errors) of n=10 trees. Plots significantly different from each other have no letter in common.

		Plot					
	Needle age	GR		KA		LI	
[mg g^{-1} dw]							
Total N	current	15.1 (0.5)	A	14.8 (0.6)	A	16.4 (0.5)	B
	1-year-old	15.3 (0.6)		15.7 (0.6)		17.3 (0.6)	
Total chlorophyll	current	2.14 (0.09)	A	2.41 (0.14)	B	2.33 (0.10)	A B
	1-year-old	2.43 (0.07)		2.72 (0.11)		2.51 (0.12)	
[mg g^{-1} Chl]							
Neoxanthin	current	48 (1)		46 (2)		47 (1)	
	1-year-old	57 (2)		55 (2)		54 (2)	
Lutein	current	128 (5)		119 (4)		120 (3)	
	1-year-old	135 (3)		130 (3)		132 (3)	
α-Carotene	current	28 (1)	A	28 (2)	A	25 (1)	B
	1-year-old	32 (2)		29 (1)		26 (2)	
β-Carotene	current	69 (3)	A	62 (2)	B	62 (2)	B
	1-year-old	66 (3)		60 (3)		57 (2)	

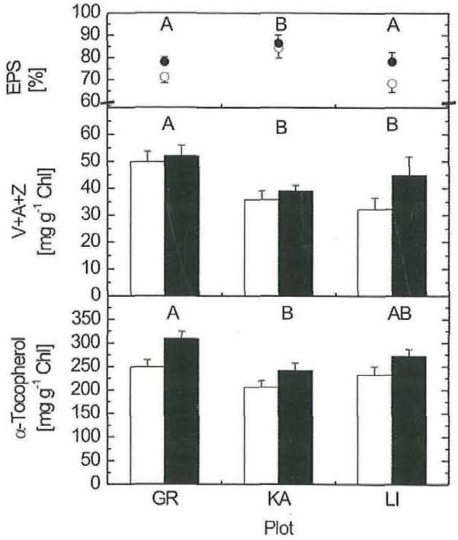

Fig. 2. α-Tocopherol and xanthophyll concentrations per g total chlorophyll (Chl), and the epoxidation state of xanthophylls (EPS=(0.5*A+V)/(V+A+Z)*100) in current (white columns and symbols) and 1-year-old (black columns and symbols) needles of *Pinus sylvestris* growing at plots in Brandenburg, Germany. V+A+Z sum of violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z). Means and standard errors of n=10 trees each. Plots significantly different from each other have no letter in common.

Total glutathione concentrations were neither affected by needle age nor by the plot (Table 2). The total glutathione pool was significantly more oxidised in 1-year-old needles compared to current ones (Table 2, Fig. 3). The glutathione redox state was similar at all plots (Table 2).

One-year-old needles had significantly higher ascorbate concentrations than current year's ones (Table 2, Fig. 3). The plot had no significant effect on total ascorbate concentrations (Table 2). The ascorbate redox state (percentage of dehydroascorbate in total ascorbate) was similar across all sampled needles (Table 2).

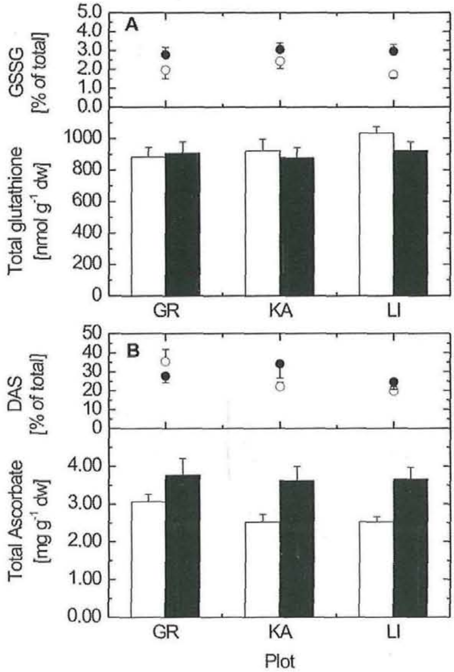

Fig. 3. Water soluble antioxidant systems in current (white columns and symbols) and 1-year-old (black columns and symbols) needles of *Pinus sylvestris* growing at field plots in Brandenburg, Germany. A. Total glutathione concentrations and redox state. B. Total ascorbate concentrations and ascorbate redox state. DAS dehydroascorbate, GSSG oxidised glutathione. Means and standard errors of n=10 trees each.

Discussion

The vegetation period 1999 was exceptionally dry and hot, and ozone concentrations exceeded recommended threshold values considerably (WIENHAUS

& al. 2002). Differences in meteorological conditions and air quality among plots were probably negligible, because the plots were close to each other and there is no strong relief to create small-scale differences. Soil differences among plots caused differences in water availability and made LI and KA comparably less xeric than GR during the months before sampling (WIENHAUS & al. 2002). Height and diameter at breast height of the trees were lowest at LI, which may be related to the long-term high N input (until 1990) by a neighbouring livestock breeding farm. Since 1990, when the farm was closed, growth rates at LI increased again (WIENHAUS & al. 2002).

As shown by needle N concentrations, the pine trees at LI had still the highest N supply with values at the upper limit of the optimum range for *Pinus sylvestris*. The Forest Foliar Coordination Centre which collects and classifies foliar nutrient concentrations of European tree species puts values of more than 17 mg g^{-1} into the highest of three classes and regards 12-17 mg g^{-1} as the normal (mid) range (FÜRST 2005). Within plots and needle age classes, needle N and chlorophyll concentrations were mostly significantly correlated (data not shown), highlighting a potential for increased light capture in N rich plants. However, when plots were compared, chlorophyll concentrations at LI (where N was highest) were intermediate between GR and KA.

The conversion state of the xanthophyll cycle is related to the light dissipation in photosynthesis, a higher EPS (= epoxidation state) means less dissipation and more light use (DEMMIG-ADAMS 2003). Plants under excess light (more than can be used in the dark reactions) often have low EPS. Trees from KA had the highest EPS, which indicates a low requirement for energy dissipation. Trees from LI and GR seemed to have a higher requirement for energy dissipation and therefore would appear comparably more stressed than those from KA. The sampling procedure ensured that all plots were sampled during the same time window of sunny days, which makes comparisons among plots valid.

Chlorophyll fluorescence analysis suggested that the differences in pigmentation and xanthophyll cycle conversion states were not related to longer-term photoinhibition (indicative of damages to the photosystems), because F_v/F_m values were around 0.80 - which is close to the maximum value of 0.83 (MAXWELL & JOHNSON 2000) - and similar among plots.

However, differences in the biochemical defence systems are mainly observed between GR on one side, and KA and LI on the other side. Needles from GR had lower total chlorophyll concentrations and higher concentrations of protective carotenoids and tocopherols per unit chlorophyll. These data correspond to higher protective capacity relative to light capture potential at the drier GR plot, a pattern that was observed previously in response to stress impacts. In contrast, there were no such differences between KA and LI, although pines at LI had higher N supply.

These results lead us to answer our research questions as follows: (1) Variations in low-molecular antioxidants ascorbate and glutathione and chloroplast pigments in pine needles among plots were not directly related to previous long-term N input (at plot LI). (2) The variation in these substances was more related to the water availability at the plots.

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