Phyton (Austria) Special issue: "Global change"	**Vol. 42**	Fasc. 3	(209)-(214)	1.10.2002

The Effects of Nitric Acid on Antioxidants and Protective Pigments in *Pinus ponderosa* Needles

By

M. TAUSZ[1)], P. E. PADGETT[2)], S. MONSCHEIN[1)] & A. BYTNEROWICZ[2)]

K e y w o r d s : *Pinus ponderosa*, nitric acid, ascorbate, glutathione, tocopherol, pigments.

S u m m a r y

TAUSZ M., PADGETT P. E., MONSCHEIN S. & BYTNEROWICZ A. 2002. The effects of nitric acid on antioxidants and protective pigments in *Pinus ponderosa* needles - Phyton (Horn, Austria) 42 (3): (209) - (214).

The antioxidants ascorbate, glutathione, tocopherol, and protective carotenoids were investigated in needles of *Pinus ponderosa* exposed to two elevated above ambient levels (with peak midday maxima of 25 and 50 ppb, respectively) of concentrations of HNO_3 for 30 days. At the 25 ppb (lower) treatment, there were increases in water soluble antioxidants ascorbate (2.78 versus 1.33 mg g^{-1} needle dry weight, P=0.015) and glutathione (759 vs. 459 nmol g^{-1} dwt, P=0.086). In the thylakoid membranes, concentrations of chlorophyll a (505 versus 907 µg g^{-1} dwt, P=0.050) decreased. Whereas carotenoids remained unchanged, α-tocopherol increased (426 vs. 388 nmol µmol^{-1} chlorophyll, P=0.050).

At the 50 ppb (higher) treatment, the increase in α-tocopherol (P=0.086; 475 nmol µmol^{-1} chl) was less pronounced and the glutathione system, the ascorbate system, and carotenoids were not significantly different from the control.

These results are consistent with the hypothesis that the major impact of high HNO_3 concentrations takes place at the needle surfaces and, possibly, in the cell walls of the mesophyll. The strongest effect was found for ascorbate, which points toward an adaptation of the antioxidant system mainly in the aqueous phase. Since responses were mainly found upon exposure to 25 ppb HNO_3, it may be speculated that upon higher concentrations the loss of antioxidant capacity counteracts defense activation. The results suggest a contribution of the antioxidative system to the plant responses to nitric acid.

[1)] Institut für Pflanzenphysiologie, Karl-Franzens-Universität Graz, Schubertstraße 51, A-8010 Graz, Austria.

[2)] Pacific Southwest Research Station, USDA Forest Service, 4955 Canyon Crest Drive, Riverside, CA 92507, United States of America.

Introduction

Nitric acid (HNO_3) vapor is a constituent of photochemical smog in urban areas. Twelve-hour daytime averages may reach as high as 18 ppb in the Los Angeles Basin (BYTNEROWICZ & al. 1998). High concentrations of HNO_3 cause significant changes in surface waxes (BYTNEROWICZ & al. 1998), and may significantly contribute to the nitrogen immissions in forest systems (BYTNEROWICZ et al. 1999). Foliar stomatal and transcuticular uptake of HNO_3 interferes with nitrogen metabolism, as determined by increases in nitrate reductase activity in pine needles following exposure. On the other hand, as with many other agents, HNO_3 can lead to the formation of reactive oxygen species (ROS) in the plant tissues (compare ELSTNER & OSSWALD 1994). Direct attack to cell wall and cell membranes may initiate ROS production, and secondary effects may lead to oxidative stress in the chloroplasts (FOYER & NOCTOR 2000). The antioxidative protection system protects the cells from adverse ROS effects (POLLE 1997), and photoprotective pigments avoid the absorbance of excessive light energy not usable in stress situations (MÜLLER & al. 2001). A preliminary short-term study exposing pine needles to acute HNO_3 levels suggested effects on the antioxidant system (BYTNEROWICZ & al. 1999).

The present study investigates responses of antioxidants and protective pigments with the objective to assess the contribution of ROS mediated processes to HNO_3 impacts on pine needles.

Material and Methods

Exposure conditions: Two year old *Pinus ponderosa* DOUGLAS ex LAWSON seedlings were exposed to atmospheric HNO_3 using continuously stirred tank reactors (PADGETT & BYTNEROWICZ 2001). A diurnal program of zero HNO_3 during the night, increasing concentrations starting at sunrise until mid afternoon, and subsequent declines to zero was designed to mimic typical ambient conditons. Plants were exposed for 30 days under high (50 ppb peak concentrations), low (25 ppb peak concentrations) and control condition.

Collection and preparation of material: Fascicles of the previous year's flush were removed and frozen in liquid nitrogen immediately. The intact needles were lyophilized and sealed in plastic bags containing a water vapor sequestering agent. Lyophilized needles were ground in a dismembrator, the needle powder was stored frozen in humidity proof plastic vials before it was subjected to HPLC analysis.

Pigment analysis: Pigments were determined on acetone extracts of the needle dry powder according to the HPLC gradient method of PFEIFHOFER 1989. Tocopherols were measured according to WILDI & LÜTZ 1996. Determination of ascorbate and dehydroascorbate was done by an isocratic HPLC method according to TAUSZ & al. 1996 and glutathione in its oxidized and reduced state was measured according to KRANNER & GRILL 1993. The concentrations of thylakoid located systems (carotenoids and tocopherol) are based on unit chlorophyll to relate their defense capacity to the light absorbance.

Statistics: Statistical evaluations were completed using Statistica (StatSoft, USA, 1994) software package. Figures show medians and median deviations which are most suitable for small sample sizes (SACHS 1992, p. 336-337). Comparisons between HNO_3 exposed samples and controls were evaluated using the Mann-Whitney test (SACHS 1992).

Results and Discussion

The lower (25 ppb) nitric acid exposure depressed chlorophyll a contents and chlorophyll a/b ratios in pine needles (Fig. 1). The chlorophyll based carotenoid concentrations and the de-epoxidation state of the xanthophyll cycle remained largely unaffected (Table 1).

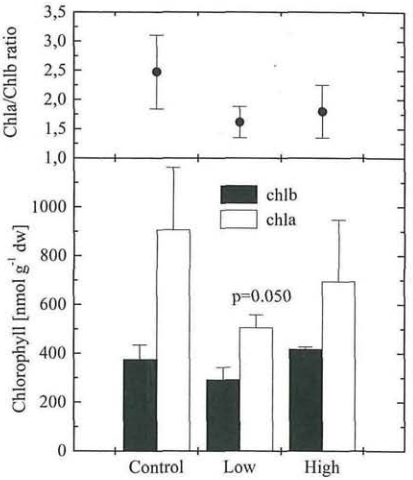

Fig. 1. Chlorophyll a and b concentrations in the 2nd flush of *P. ponderosa* of needles exposed to atmospheric HNO_3. Significance levels are presented for Mann-Whitney-test comparison to the control. Data are medians and median deviations of N=5 replicate trees.

Table 1. Carotenoids and α-tocopherol in the previous year's *P. ponderosa* needles [nmol μmol^{-1} total $chlorophyll^{-1}$]. Medians ± median deviations of 5 individual trees. P-values indicate significance level for differences from the control (P>0.1 not shown). V = violaxanthin, A = antheraxanthin, Z = zeaxanthin, De-epoxidation state = (Z+0.5*A)/ (V+A+Z)*100.

	Control	Low (25 ppb HNO_3)	High (50 ppb HNO_3)
Neoxanthin	73 ± 13	92 ± 3	87 ± 13
		P=0.086	
Lutein	239 ± 33	284 ± 47	264 ± 37
V+A+Z	62 ± 11	56 ± 11	63 ± 18
α-*Carotene*	38 ± 3	36 ± 4	34 ± 5
β-*Carotene*	109 ± 25	111 ± 5	128 ± 12
α-*Tocopherol*	388 ± 28	426 ± 23	475 ± 50
		P=0.050	*P=0.086*
De-epoxidation state [%]	59 ± 5	52 ± 5	63 ± 7

The main lipophilic antioxidant, α-tocopherol, increased upon the HNO_3 treatments (Table 1).

The concentrations of water soluble antioxidants, glutathione and ascorbate, increased in needles of pine trees exposed to 25 ppb HNO_3. This effect was not significant in the 50 ppb treatment (Fig. 2 and 3). The redox states of ascorbate pools did not change significantly (Fig. 2), whereas the percentage of oxidized glutathione in the glutathione pool was significantly lower in needles of trees exposed to 25 ppb HNO_3 (Fig. 3).

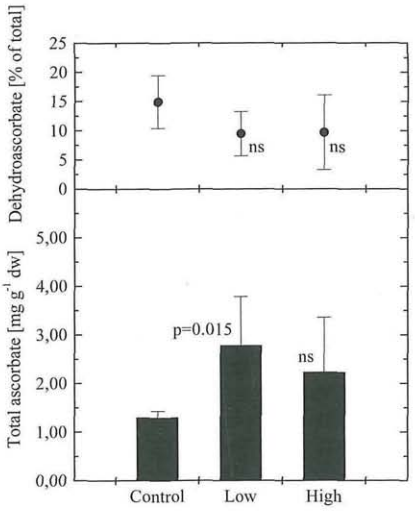

Fig. 2. The ascorbate system in the 2nd flush of *P. ponderosa* needles exposed to atmospheric HNO_3. Significance levels are presented for Mann-Whitney-test comparison to the control. Data are medians and median deviations of N=5 replicate trees.

Increased pool sizes of glutathione and ascorbate may confer an increased defense capacity, underlined by a more strict control of the reduced state of the glutathione pools. Corresponding results were found for many other stress impacts (including air pollutants) on conifers (POLLE 1997). A decrease in chlorophyll contents together with a relative increase in the thylakoid based α-tocopherol adds to the picture of increased defense potency against ROS. However, the unaffected de-epoxidation state of the xanthophylls showed that direct light protection was not activated by HNO_3 treatment. These data are consistent with the hypothesis that the major impact of HNO_3 takes place at the needle surface and, after stomatal uptake, in the cell walls of the mesophyll. Hence, ROS production may be due to attack at the plasmalemma and antioxidant responses in the adjacent cytoplasm, which would rely on the ascorbate and glutathione system. Effects on thylakoid based systems would occur only after secondary products reach the chloroplasts.

Furthermore, the uptake of nitric acid vapor will result in increased nitrate concentrations in the mesophyll cell walls. For nitric oxides it was shown that chemical reduction of nitrate by apoplastic ascorbate contributes considerably to the observed high uptake rates (HABERER & al. 2001), a mechanism that will also work with HNO_3. Apoplastic ascorbate will be oxidized to dehydroascorbate and most probably has to be re-translocated into the symplasm to be regenerated in the ascorbate-glutathione cycle (HOREMANS & al. 2000). In this case, increased tissue ascorbate and glutathione concentrations will facilitate the accelerated ascorbate turnover across the plasmalemma.

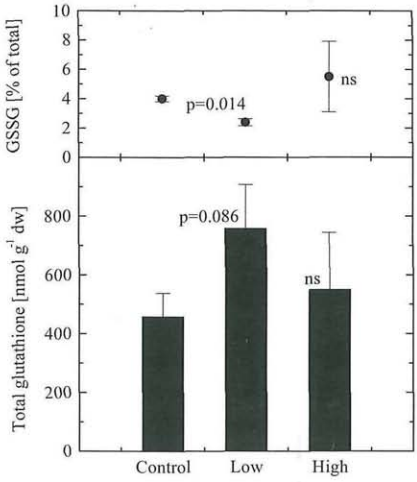

Fig. 3. The glutathione system in the 2nd flush of *P. ponderosa* needles exposed to atmospheric HNO_3. Significance levels are presented for Mann-Whitney-test comparison to the control. Data are medians and median deviations of N=5 replicate trees.

However, the observed effects were only pronounced in needles exposed to the lower HNO_3 concentration, whereas the higher concentrations resulted in no significant differences from the controls. Possibly, like for many other stress impacts, the defense responses follow an optimum curve, and the loss of defense capacities upon 50 ppb (developing damage) counteracts the activations observed in needles exposed to 25 ppb, which may be a transient phenomenon.

The present results show changes in the antioxidant defense sytems in ponderosa pine needles upon atmospheric HNO_3 impact and, hence, a contribution of ROS mediated processes.

Acknowledgement

The work was supported by a grant of the Fulbright Commission to M. TAUSZ and the USDA-NRI grant #97-35100-4402.

References

BYTNEROWICZ A., PERCY K., RIECHERS G., PADGETT P. & KRYWULT M. 1998. Nitric acid vapor on forest trees - deposition and cuticular changes. - Chemosphere 36: 697 - 702.

— , PADGETT P., PERCY K., KRYWULT M., RIECHERS G. & HOM J. 1999. Direct effects of nitric acid on forest trees. - In: MILLER P. R. & MCBRIDE J. R. (Eds.), Oxidant air pollution impacts in the montane forests of southern California. A case study of the San Bernardino mountains. Ecological Studies 134. - Springer-Verlag, New York.

ELSTNER E. F. & OSSWALD W. 1994. Mechanisms of oxygen activation during plant stress. - Proceedings of the Royal Society of Edinburgh 102B: 131 - 154.

FOYER C. H. & NOCTOR G. 2000. Tansley review No. 112. Oxygen processing in photosynthesis: regulation and signalling. - New Phytol. 146: 359 - 388.

HABERER K., JAEGER L. & RENNENBERG H. 2001. Apoplastic ascorbate influences the stomatal uptake of nitrogen dioxide (Meeting abstract). - 5th International symposium on "Responses of plant metabolism to air pollution and global change", Puławy, Poland, November 1 - 4, 2001. Programme and Abstracts Volume p. 38.

HOREMANS N., FOYER C. H. & ASARD H. 2000. Transport and action of ascorbate at the plant plasma membrane. - Trends in Plant Science 5: 263 - 267.

KRANNER I. & GRILL D. 1993. Content of low-molecular-weight thiols during the imbibition of pea seeds. - Physiol. Plant. 88: 557 - 562.

MÜLLER P., LI X.-P. & NIYOGI K. K. 2001. Non-photochemical quenching. A response to excess light energy. - Plant Physiol. 125: 1588 - 1566.

PFEIFHOFER H. W. 1989. Evidence of chlorophyll b and lack of lutein in *Neottia nidus-avis* plastids. - Biochem. Physiol. Pflanzen 184: 55 - 61.

POLLE A. 1997. Defense against photooxidative damage in plants. - In: SCANDALIOS J. G. (Ed.), Oxidative stress and the molecular biology of antioxidant defenses, pp. 623 - 666. – Cold Spring Harbor Laboratory Press, New York.

PADGETT P. E. & BYTNEROWICZ A. 2001. Deposition and absorption of the air pollutant HNO_3 vapor to soil surfaces. - Atmos. Environ. 35: 2405 - 2415.

SACHS L. 1992. Angewandte Statistik. 7. Aufl.- Springer Berlin, Heidelberg, New York.

TAUSZ M., KRANNER I. & GRILL D. 1996. Simultaneous determination of ascorbic acid and dehydroascorbic acid in plant materials by high-performance liquid chromatography. - Phytochem. Analysis 7: 69 - 72.

WILDI B. & LÜTZ C. 1996. Antioxidant composition of selected high alpine plant species from different altitudes. - Plant Cell Environ. 19: 138 - 146.