Ascorbic Acid and Glutathione Contents of Spruce Needles from Different Locations in Bavaria

W. F. Osswald, H. Senger, and E. F. Elstner

Institut für Botanik und Mikrobiologie, Lehrstuhl für Botanik der Technischen Universität München, Arcisstraße 21, D-8000 München 2, Bundesrepublik Deutschland

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Green and bleached needles or needle segments were analyzed for their ascorbic acid and glutathione contents. All bleached needles or segments exhibited higher amounts of both substances as compared to green controls.

The physiological significance of the increase in ascorbic acid and in glutathione is discussed in content with their roles as antioxidants.

Introduction

In 1980 a "mysterious" spruce decline was observed for the first time at higher altitudes in the East Bavarian mountains (Fichtelgebirge and Bavarian Forest). As a typical symptom, the oldest needles became chlorotic with the exception of the current year needles [1] a symptom which has never been described in earlier times. In most cases light exposed needles or needle segments exhibit stronger symptoms as compared to shaded needles.

The gaseous air pollutant SO_2 and photooxidants with ozone as the main component are discussed as possible parameters in spruce decline [2, 3]. Sulfur dioxide may display its toxic activity during the photooxidation of sulfite to sulfate within the chloroplasts. Sulfite radicals and reactive oxygen species may be formed which can start lipidperoxidation and pigmentcooxidation [4, 5]. Furthermore the toxic effect of SO_2 may also involve the inhibition of the enzyme ribulose-1,5-bisphosphate-carboxylase [6].

Ozone toxicity involves either catalysis of free radical formation [7] or ozone molecules themselves [8, 9] causing lipidperoxidation and pigmentcooxidation [10].

Within the stroma of the chloroplasts a detoxification cycle is localized which reduces different reactive oxygen species to water at the expense of NADPH *via* ascorbic acid and glutathione [11]. Several workers demonstrate that different conifers as well as herbaceous plants increase their antioxidants after fumigation with SO_2 or O_3 [12–15]. Our intention was to investigate whether the content of antioxidants in needles and needle segments of damaged and healthy trees exhibits significant differences, possibly allowing conclusions concerning the primary damaging factors or events.

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Experimental Procedures

Materials

The HPLC gradient system was from Beckman (München, FRG) consisting of a variable wavelength detector (Type 165), or a fluorescence detector, two pumps (Type 112), Organizer (Type 340), Controller (Type 420) and printer (Type C-R2AX) in combination with an analytical Reversed-Phase-Column (ODS-ULTRASPHERE, 5 μm, 250 mm × 4.6 mm).

The spruce needle samples from the Bavarian Forest were taken at the "Hochstraße", and needles from the Fichtelgebirge were collected at the "Scheeberg". The mixed needle samples were collected in November 1985; whole branches were harvested in April 1986.

Ascorbic acid and glutathione were purchased from Sigma, München. The chlorophyll determination was performed using a Kontron UVIKON 810 spectrophotometer (Eching, München).

Methods

Collection of spruce needles

Six "symptom bearing" (visibly damaged) and six healthy trees were used for the "mixed" spruce needle samples at each location. The needles were plucked off under liquid nitrogen, transported in dry ice and stored at −80 °C in the lab-freezer.

For investigations on needle segments the whole branch was cut off, put into a nutritive solution with the cut end [16] and stored in a cooling chamber at 4 °C.

We have chosen damaged trees which exhibited branches with bleached as well as green needles. Additionally, branches of a completely green tree in the direct neighborhood of the damaged trees were also collected. The green needles of the damaged trees are called "internal control" and those of the neighbouring trees "external controls".

Extraction and determination of the antioxidants

Extraction and determination of ascorbic acid and of glutathione: Approximately 0.5 g of needles were ground in a mortar with liquid nitrogen. The frozen powder was then extracted with 5 ml potassium phosphate buffer (100 mM, pH = 2.5) for 3 min. The extract was filtered and an aliquot of 5 µl was used for HPLC analysis. Ascorbic acid was monitored at 263 nm.

Glutathione was derivatized with orthophthaldialdehyde (OPA) [17]. For glutathione determination a fluorescence detector (excitation 360 nm/emission 455 nm) and for the ascorbic acid detection a variable wavelength detector (263 nm) was used.

Potassium phosphate buffer (20 mM, pH = 6.5) was useful as the mobile phase (flow rate 1 ml/min) for the ascorbic acid determination. For the glutathione detection we used a mixture of potassium phosphate solution (20 mM, pH = 7.2) and methanol (85:15, v/v).

Ascorbic acid and glutathione were determined as referred to authentic standards (Sigma, München). Two separate determinations were performed for each needle sample. The deviation between parallel runs was smaller than 5%.

Determination of chlorophyll

For chlorophyll determination the needles were powdered in liquid nitrogen. The chlorophyll content was measured according to Arnon [18].

Determination of dry weight

Frozen needles (liquid nitrogen) were powdered in a mortar, dried at 60 °C for three days and weighted with an analytical balance (Satorius Type).

Results

The chlorophyll contents of the needle tip, the middle part of the needle and of the needle base of healthy and damaged spruce needles (see Table I)

Green needles of the external as well as of the internal control exhibit comparable values. In most cases the chlorophyll is uniformly distributed within the whole needle. Sometimes a small chlorophyll in-

Table I. The chlorophyll content of different needle segments of healthy and damaged spruce needles.

		Chlorophyll [mg/g d.w.]								
		Tip			Middle part			Base		
	Needle year	External control	Internal control	Damaged needle	External control	Internal control	Damaged needle	External control	Internal control	Damaged needle
Bavarian Forest (Hochstraße)	1985	2,1*	2,6	1,6	2,3	2,9	1,8	2,4	2,9	2,0
	1984	2,4	3,2	1,1	2,6	2,9	1,6	2,8	3,5	2,0
	1983	2,7	3,3	0,8	2,8	3,3	1,0	2,8	3,5	1,4
	1985	2,1	1,9	1,7	2,3	1,8	1,7	2,3	1,9	1,8
	1984	2,6	2,4	0,8	2,6	2,4	1,2	2,7	2,4	1,6
	1983	2,8	2,6	0,8	2,9	2,7	1,0	2,9	2,7	1,3
Fichtelgebirge	1985	1,6	2,1	1,6	1,7	2,3	1,4	1,7	2,3	1,6
	1984	2,2	2,6	0,9	2,2	2,7	0,9	2,4	2,8	1,3
	1983	2,6	2,2	0,7	2,7	2,8	0,8	2,6	2,7	1,0
	1985	1,9	1,6	1,0	2,1	1,7	1,2	2,0	1,7	1,4
	1984	2,6	1,9	0,7	2,7	2,1	0,7	2,5	2,0	0,8
	1983	2,9	2,7	0,7	3,1	2,6	0,8	2,8	2,4	0,6

* Mean of two separate determinations.

crease can be found from the tip to the base of the needle.

All damaged spruce needles exhibit a strong chlorophyll decrease as compared to control needles. In all cases the needle tip shows the smallest chlorophyll content. Furthermore the chlorophyll values of damaged spruce needles decrease with the needle age. In most cases the oldest damaged spruce needles are uniformly bleached. The current year needles still appear green but their chlorophyll content is already lower as compared to control needles.

The ascorbic acid contents of the needle tip, the needle middle part and of the needle base of healthy and damaged spruce needles (see Table II)

The ascorbic acid contents of green control needles slightly increases from the needle base to the needle tip. The ascorbic acid content also increases within damaged needles from the green base up to the yellow tip; this increase in most cases is percentually higher as compared to control needles.

In each case all bleached needle parts exhibit higher ascorbic acid contents as compared to green control needle segments. The strongest increase can be found in bleached needle segments of two- and three-year-old needles. For those needles the increase in ascorbic acid differs between 45 and 350%.

No or only a small increase in ascorbic acid can be found in segments of current year needles which exhibit only small differences in their chlorophyll contents as compared to green controls.

The glutathione content of the needle tip, the middle part of the needle and of the needle base of healthy and damaged spruce needles (see Table III)

The glutathione contents of the control needles increase slightly from the needle base up to the needle tip. Within damaged needles we find the same trend. In each case all needle segments of damaged needles exhibit higher glutathione contents as compared to green controls. The increase in glutathione ranges from 10 to 184%.

A comparison of the chlorophyll, ascorbic acid and glutathione contents of the upper and of the lower side of damaged and green control needles (see Table IV)

Damaged and green control needles were collected from the same tree. The upper and lower side of the control needles exhibit similar chlorophyll, glutathione as well as ascorbic acid contents.

The upper side of all damaged needles shows much smaller chlorophyll contents as compared to the lower sides. Also lower chlorophyll contents are measured for the upper side of two- and three-year-old damaged needles as compared to green controls.

In each case all upper parts of damaged needles exhibit higher ascorbic acid and glutathione contents

Table II. The ascorbic acid contents of different needle segments of healthy and damaged spruce needles.

		Ascorbic acid [mg/g d.w.]								
		Tip			Middle part			Base		
	Needle year	External control	Internal control	Damaged needle	External control	Internal control	Damaged needle	External control	Internal control	Damaged needle
Bavarian Forest (Hochstraße)	1985	1,7*	1,8	2,5	1,8	2,0	2,3	1,5	2,0	2,0
	1984	1,3	1,4	4,3	1,4	1,0	2,7	1,5	0,8	2,1
	1983	1,5	0,8	3,6	1,6	0,9	2,9	1,5	0,9	2,8
	1985	1,9	2,6	2,9	1,8	2,4	2,6	1,7	2,3	2,4
	1984	1,7	1,6	3,4	1,7	1,4	2,7	1,8	1,4	2,3
	1983	1,5	1,5	3,7	1,5	1,3	2,8	1,6	1,2	2,4
Fichtelgebirge	1985	2,4	3,2	4,8	1,9	2,9	3,6	1,7	2,6	3,5
	1984	3,1	2,6	5,4	2,8	2,8	4,9	2,4	2,9	4,3
	1983	2,1	3,4	6,1	1,6	3,6	5,2	2,5	3,1	5,1
	1985	2,2	3,6	7,3	1,6	2,8	5,1	1,6	2,4	4,7
	1984	2,4	3,8	7,8	2,3	3,1	6,4	2,1	3,2	6,1
	1983	2,6	2,6	5,9	2,1	2,6	5,3	1,9	2,4	4,6

* Mean of two separate determinations.

Table III. The glutathione contents of different needle segments of healthy and damaged spruce needles.

	Needle year	Tip External control	Tip Internal control	Tip Damaged needle	Middle part External control	Middle part Internal control	Middle part Damaged needle	Base External control	Base Internal control	Base Damaged needle
		Glutathione [µg/g d.w.]								
Bavarian Forest (Hochstraße)	1985	105*	131	151	100	126	127	101	118	119
	1984	132	154	218	127	146	211	129	137	171
	1983	184	198	287	201	184	243	193	165	211
	1985	131	157	188	111	143	173	107	145	171
	1984	167	183	244	156	171	211	158	170	201
	1983	187	207	301	182	201	233	193	197	220
Fichtelgebirge	1985	154	171	328	128	162	278	131	164	259
	1984	192	161	431	164	154	311	153	157	305
	1983	274	319	509	232	297	455	208	244	431
	1985	211	240	320	205	214	280	201	199	266
	1984	311	421	531	286	287	487	274	244	310
	1983	196	367	541	290	299	463	297	287	421

* Mean of two separate determinations.

Table IV. The chlorophyll, ascorbic acid and glutathione contents of the upper and lower side of healthy and damaged spruce needles.

	Needle age 1985				Needle age 1984				Needle age 1983			
	Green control needles		Damaged needles		Green control needles		Damaged needles		Green control needles		Damaged needles	
	Upper side	Lower side	Upper side	Lower side	Upper side	Lower side	Upper side	Lower side	Upper side	Lower side	Upper side	Lower side
Chlorophyll [mg/g d.w.]	2,0*	2,3	1,6	2,3	2,6	2,7	0,9	1,6	2,5	2,6	0,8	1,5
Ascorbic acid [mg/g d.w.]	1,6	1,5	2,7	2,1	1,2	1,3	3,2	2,4	1,4	1,4	2,9	2,1
Glutathione [µg/g d.w.]	132	126	151	127	147	139	257	211	166	174	280	237

* Mean of two separate determinations.

as compared to all green control segments in damaged or healthy needles.

The ascorbic acid and glutathione contents of green and bleached needle segments in direct neighbourhood to necrotic spots (see Table V)

In each case higher ascorbic acid as well as glutathione concentrations can be measured in bleached as compared to green needle segments. All necrotic spots which were caused by different fungi (data not shown) were cut-off before bleached and green needle segments were used for analysis.

A comparison of the chlorophyll, ascorbic acid and glutathione contents of green and bleached mixed needle samples (see Table VI)

In each case all samples of bleached needles exhibit lower chlorophyll contents but higher ascorbic acid and glutathione concentrations as comparable controls.

Table V. The ascorbic acid and glutathione contents of green and bleached needle segments adjacent necrotic spots.

	Needle age 1985		Needle age 1984		Needle age 1983	
	Green needle segment	Bleached needle segment	Green needle segment	Bleached needle segment	Green needle segment	Bleached needle segment
Ascorbic acid [mg/g d.w.]	1,7*	2,4	1,6	2,9	1,3	2,3
Glutathione [μg/g d.w.]	98	157	151	193	180	211

* Mean of two separate determinations.

Table VI. The chlorophyll, ascorbic acid and glutathione contents of green and bleached mixed needle samples.

		Needle age 1984		Needle age 1983		Needle age 1982		Needle age 1981	
		Control needles	Damaged needles	Control needles	Damaged needles	Control needles	Damaged needles	Control needles	Damaged needles
Bavarian Forest	Chlorophyll [mg/g d.w.]	2,2*	2,0	2,7	1,7	3,2	0,7	3,3	0,65
	Ascorbic acid [mg/g d.w.]	2,5	5,5	4,0	5,8	5,6	7,9	5,2	6,9
	Glutathione [μg/g d.w.]	12	18	30	152	25	75	22	206
Fichtelgebirge	Chlorophyll [mg/g d.w.]	3,5	2,7	3,0	2,0	3,2	1,5	3,1	1,4
	Ascorbic acid [mg/g d.w.]	6,5	8,0	6,6	14,8	5,9	11,8	6,0	13,0
	Glutathione [μg/g d.w.]	275	362	264	382	243	312	260	364

* Mean of two separate determinations.

Discussion

In this paper we compare the ascorbic acid and glutathione contents of damaged needles and needle segments with the respective green controls. Within damaged needles we found an increase in ascorbic acid as well as in glutathione from the green needle base up to the bleached needle tip. At the same time all chlorotic segments of damaged needles exhibited higher amounts of ascorbic acid and of glutathione as compared to corresponding segments of green needles of the internal or of the external controls. This result holds in most cases for one- and two-year-old needles. For current year needles which exhibit no or only small differences in their chlorophyll contents from the base to the tip we generally found only marginal differences in these antioxidants.

In most cases the increase in ascorbic acid was considerable higher as compared to the glutathione enhancements. We could not find any differences for bleached and green needles harvested either from the Bavarian Forest or from the north-east of Bavaria (Fichtelgebirge). No differences could be found for the respective oxidized forms, DHAsc or GSSG, in green and bleached needles (data not shown). The increase in the concentrations of the two antioxidants can be taken as evidence for an enhanced demand for detoxification capacity towards reactive oxygen species which may by formed in the chloroplast during the bleaching process [19]. This explanation will lead to the following contradiction however: Why are needle segments bleached, which obviously contain such high amounts of antioxidants?

This question may be answered in the following way: The most important reactive oxygen species which initiates pigmentoxidation under conditions of "overreduction" of the electron transport is singlet oxygen [20]. Singlet oxygen is formed in the thylakoid membranes in direct neighbourhood of chlorophyll molecules [21]. The antioxidants ascorbic acid and glutathione are localized in the chloroplast stroma and do not contact singlet oxygen. On the other hand these antioxidants may detoxify other reactive oxygen species such as superoxid and hydrogenperoxid which are formed on the thylakoid surface. Thus further destructive reactions such as the breakdown of the outer chloroplast membrane will not occur [22].

It has been shown by different workers that ascorbic acid as well as glutathione increase in conifer needles after prolonged fumigation with SO_2 and or with O_3 [12, 13]. On the other hand treatment with sulfate enhances the GSH-contents in needles [23].

On the basis of these results we draw the conclusion that the measured increase in ascorbic acid and in glutathione may be caused by the chronic influence of different air pollutants such as SO_2 and/or photooxidants. Furthermore it is of great interest that we found enhanced ascorbic acid and glutathione contents in bleached needle segments in direct neighbourhood of necrotic spots which were undoubtedly caused by fungal infection (data not shown).

In future experiments it should be elucidated whether fungal infection can also lead to an increase in these antioxidants in spruce needles.

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