Impact of Aminotriazole and Paraquat on the Oxidative Defence System of Spruce Monitored by Monodehydroascorbic Acid

A Test Assay for Oxidative Stress Causing Agents in Forest Decline

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Treatment of young spruces with the herbicides aminotriazole and paraquat leads to oxidative stress resulting in a drastically increased production of ascorbic acid radical (monodehydroascorbic acid, MDAA) which could be demonstrated by *in vivo* EPR-analysis. While paraquat action is accompanied by a rapid decrease in reduced ascorbic acid content, no change in ascorbic acid content was observed by aminotriazole treatment. Thus MDAA-measurements can undoubtedly verify an existing oxidative stress of different origin in spruce needles. A decision between both radical generating mechanisms based on the radical kinetics is possible.

Introduction

During investigations on forest decline details of conifer physiology became of great interest. As a complex disease forest decline obviously involves oxidative stress which finally causes oxidative damage of needles (bleaching of pigments) [1]. In this context investigations on antioxidative mechanisms in photosynthetic tissues are of great interest. The ascorbic acid redox system is a powerful part of the antioxidative system in plant leaves [2]. Ascorbic acid can detoxify most of the reactive oxygen species of enzymatic or non-enzymatic origin [3, 4]. Hydrogen peroxide (H_2O_2) is one of the most important toxic oxygen species. It was detected in fog and rain up to concentrations of 100 μM, furthermore it is produced in plants by several pathways [5, 6].

In field studies we found an excessive radical production in spruce trees and spinach plants growing in the neighbourhood of Amitrol treated crop fields [7]. The radical formed was identified as monodehydroascorbic acid (MDAA), the first product of oxidation of ascorbic acid [7].

The investigations reported here are a detailed analysis of the action of two herbicides which influence the formation of reactive oxygen species (particularly hydrogen peroxide) and their responses to the antioxidant ascorbic acid in spruces.

One of these herbicides, paraquat, is a member of the methylviologens and well known as an uncoupling agent in photosynthetic electron transport [4, 8]. It leads to an increased reduction of molecular oxygen yielding superoxide radical ($O_2^{\cdot-}$). This radical is detoxified by superoxide dismutase which is present in chloroplasts yielding hydrogen peroxide and oxygen [6]. The resulting H_2O_2 is detoxified in chloroplasts by ascorbate peroxidase in the "ascorbate-glutathione cycle" [4]. The first product is MDAA, which finally is regenerated to ascorbic acid with NADPH from the photosynthetic electron transport [9].

The second herbicide we investigated was 3-amino-1,2,4-triazole (aminotriazole), the active substance of Amitrol. This heterocyclic compound is known to be an inhibitor of catalase action [10]. The main function of catalase which is absent in chloroplasts is the detoxification of H_2O_2 generat-

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ed in peroxisomes as one product of photorespiration. Photorespiration takes place under high light and high temperature conditions and is due to action of ribulosebisphosphat carboxylase/oxygenase which is able to fix oxygen instead of carbondioxide [11]. The subsequent reaction sequence results in production of H_2O_2 and loss of CO_2 [6].

In this study we examined the action of paraquat and aminotriazole on reduced ascorbic acid concentration and simultaneous *in vivo* production of MDAA in needles of seven year old cloned spruce trees growing under field conditions.

Materials and Methods

Plant material

Cloned spruce saplings (*Picea abies* [L.] Karst), (seed origin Black Forest) were planted in pots in a home mix of natural soil with some fertilizer in the fifth year of growth. The trees were grown in slight shadow under field conditions in the Black Forest near Freiburg. One month before the herbicide treatment started the plants were transferred to full sun light at a place near Tübingen.

Herbicide treatment

Two of these trees were entirely treated with 1% aqueous solution of paraquat or 3-amino-1,2,4-triazole, using a spray device. A contamination of the soil was avoided by temporary sealing the pot surface. Small twigs were harvested over a period of several months and immediately subjected to ascorbic acid determination or EPR investigations, respectively.

Ascorbic acid determination

At harvest needles plucked off the twigs were frozen in liquid nitrogen. For analysis 30–60 mg of frozen, one year old needles were pulverized in a microdismembrator (Braun, Melsungen, Germany) in teflon cells containing tungstencarbid balls at liquid nitrogen temperature. 3 ml of 2% metaphosphoric acid (Merck, Germany) were added and the components were mixed. After thawing the mixture was centrifuged (4 °C, 20 min, 18000 × *g*). The clear supernatant was used for direct analysis of ascorbic acid.

The determination was modified after the classical method of Tillmanns and is based on the reduction of 2,4-dichlorophenolindophenol (DCPIP) by ascorbic acid [12]. 0,5 ml sodiumacetate (60 mM, pH 5) were added to 0,5 ml extract to adjust the pH to 2,5.

Subsequently 2 ml of DCPIP solution (0.2 mM) were added, and the extinction at 520 nm (Uvikon 930, Kontron analytics) was measured immediately. 50 µl of ascorbic acid solution (1%) was added to bleach the dye completely, to avoid interference extinction from other components. For calibration test series with external ascorbic acid standard solutions in a range from 0 to 450 µM were run.

Monodehydroascorbic acid determination by EPR measurements [13]

One year old needles were pulled off the branches immediately before measuring and placed with the back side on a quartz plate with the help of Pelican glue which is EPR inactive in the dark and under illumination. The quartz plate was located in a Bruker EPR-X-band cavity. The measurements were done at room temperature using a Bruker ESP 300 spectrometer, by accumulation of 50 scans using a FF-lock under the following conditions: microwave energy 5 mW, 10 kHz field modulation, modulation amplitude 400 m (range 50 G, gain 1.0×10^5, time constant 82 ms). The *g*-values were determined by comparison with the standard 2,6-di-tert-butyl-4-tert-butoxy-phenoxyl (g = 2.00463). As light source a Schott Kaltlichtquelle KL 1500 was used. The assignment of the different signals observed to the radical monodehydroascorbic acid (MDAA) was done by interpretation of the hyperfine structure observed under high resolution conditions (not shown) and with the help of the g-factors. The limit of error is estimated bo be less than 2×10^{-4}. The relative MDAA-concentrations were calculated by dividing the peak-to-peak intensities obtained from the difference spectra light-dark, by the peak-to-peak intensity of photosignal I. The reproducibility of the values obtained depends mainly on the signal-to-noise ratio. Typical values are 100:3.

Results

Both herbicides led within 2 days to a drastic increase in the production of MDAA. Six days after paraquat treatment the measured concentration of MDAA was over 50-fold higher than in untreated controls. This maximum was followed by a rapid

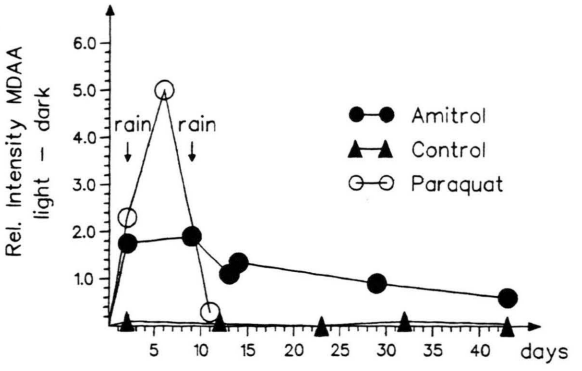

Fig. 1. Intensity of MDAA *versus* the time after treatment with herbicides.

decrease in MDAA production and after 2 weeks no signal could be detected anymore (Fig. 1). This finding corresponded to the bleaching and loss of needles. Some buds opened and grew for a short time, but after 4 weeks the whole tree died.

The treatment with aminotriazole as well resulted in a significant increase in MDAA production with almost constant concentrations over a period of 10 days. However, in contrast to paraquat (s. Fig. 1) the relative intensities are remarkably smaller and above all, the diminution of the MDAA radical is considerably slower indicating a persistent but not lethal impact on the photosystem. These data were obtained from spectra shown in Fig. 2, for example. The control tree (a) exhibits merely small MDAA concentration due to the well-known Mehler-reaction. Treatment with aminotriazole (b) leads to a drastic increase of the two MDAA lines at the low side of the photosignal I. Their peak to peak intensities dominate the EPR-spectrum at this time. After 10 weeks (c) concentration of MDAA was still 5-fold higher as compared to the control. The almost undisturbed photosignal I (d) was observed not before 4 months past contamination. Bleaching of needles from the

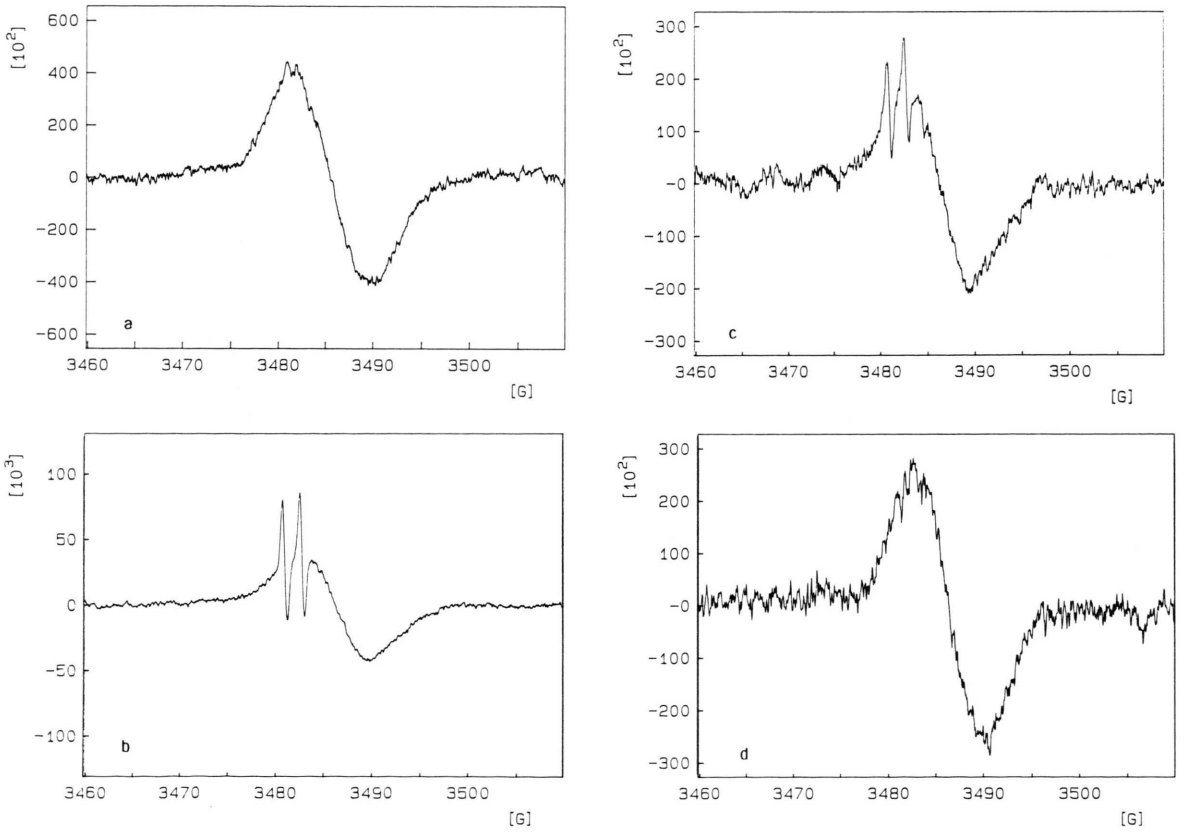

Fig. 2. EPR-spectra of spruce needles tree; a: control; b: 2 weeks after treatment with 1% aminotriazole; c: 10 weeks after treatment; d: 16 weeks after treatment.

sunny side especially took place but no loss of needles was observed. Bud break was normal but the untreated young needles in some parts showed pigment bleaching (Fig. 3).

The content of ascorbic acid differed drastically in the two trees. From the beginning, paraquat treatment leads to a rapid loss of ascorbic acid, after 10 days less than 5% of the control was found (Fig. 4). On the other hand no change in reduced ascorbic acid content was found in the aminotriazole treated spruce. The content of ascorbic acid was within the same range as in the control over a period of 7 weeks.

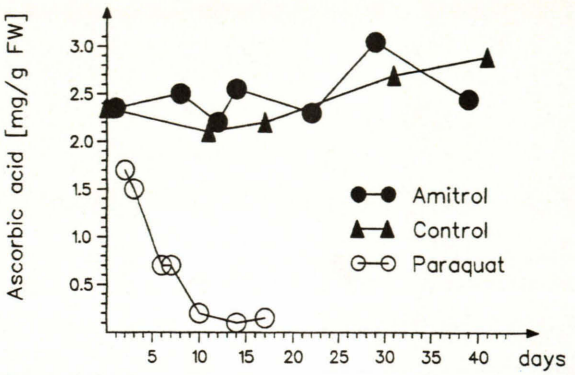

Fig. 4. Concentration of ascorbic acid *versus* the time after treatment with herbicides.

Discussion

The treatment of young spruce trees with the herbicides paraquat and aminotriazole resulted at first glance in comparable oxidative stress, as demonstrated by the measurement of MDAA.

While the paraquat treated spruce died after a few weeks preceded by bleaching and loss of needles, the aminotriazole treated spruce showed remarkable symptoms of destructive stress under the conditions applied but there after a full regeneration took place. An explanation for the difference in development after comparable primary effects is given by the content of reduced Vitamin C. In the paraquat treated spruce tree a fast decrease in ascorbic acid concentration was found which implicates that no regeneration of oxidized ascorbic acid occurs. Regeneration of used ascorbic acid needs electrons from an intact photosynthetic electron transport essentially *via* NADPH. This path-

Fig. 3. Branch of a spruce tree; right: 7 weeks after treatment with 1% aminotriazole, left: control.

way, however, is interrupted by paraquat. Therefore the regeneration of oxidized ascorbic acid stopped after loss of the NADPH-pool [4]. The paraquat-induced O_2^--production and subsequent H_2O_2-formation which now could not be effectively detoxified led to a strong and non-compensated oxidative action which resulted in tree senescence.

Treatment with the catalase inhibitor 3-amino-1,2,4-triazole likewise resulted in a great production of MDAA, but not in a loss of reduced ascorbic acid. In this case the regeneration of ascorbic acid is obviously effective (Fig. 4). Inhibition of catalase which does not involve ascorbic acid for the antioxidative action, however, induces ascorbate peroxidase as was demonstrated for *Ricinus communis* [14]. This enzyme detoxifies H_2O_2 instead of catalase by oxidation of ascorbic acid. We assume this mechanism to be active as an explanation for the production of MDAA in this case.

But why does reduced ascorbic acid content not change? Aminotriazole had no effect on the photosynthetic electron transport, so NADPH can be produced. This, however, enabled the regeneration of oxidized ascorbic acid by the other enzymes of the "ascorbate-glutathione cycle". Nevertheless the detoxification of H_2O_2 from photorespiration seems to be not as effective with ascorbic acid as with catalase because even after a single treatment there were visible signs of bleaching over a period of several months. The probability that plants have different capacity to replace the catalase action after inhibition with aminotriazole could be the reason for specific effects of the herbicide.

During the last years some attempts were made to establish the ascorbic acid content of needles as a marker for early diagnosis of forest decline [1, 15]. Our investigations showed that only MDAA-production indicated the action of both substances which interfered with the health of the trees. MDAA-measurements were much more sensitive than ascorbic acid concentration when the antioxidative ascorbic acid system is involved in stress response. Changes in the great ascorbic acid pool – up to 50 mM in chloroplasts [16] – needed more time, particularly when regeneration takes place, to become detectable as the *in vivo* production of MDAA which is a direct indicator for ascorbic acid oxidation. The observation of the ascorbic acid radical MDAA is not sufficient to decide between an interaction of the pollutant with the electron transport chain (paraquat) or the enzyme catalase (aminotriazole). However, kinetic investigations on the rise and decay of the radical intensity in dependence of illumination of the needles could provide decisive information. Preliminary investigations of the complex mechanisms indicate at least a two orders of magnitude faster kinetics if an impact on the electron transport is responsible for MDAA-production. Investigations in order to clarify the complicated kinetic behavior are in progress, for both conifers and deciduous plants.

Considering the possibility, that unidentified anthropogenic airborn trace substances are involved in forest decline the EPR-measurement of MDAA-production might be very well suited to test potential candidates under controlled *in vivo* conditions when the antioxidative ascorbic acid system is involved [17], as was demonstrated by the different action of aminotriazole and paraquat.

Acknowledgements

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