

Influence of the Herbicides Amitrole and Norflurazon on Greening of Illuminated Potato Microtubers

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Potato microtubers turn green within a few days when kept in the light. The initial phases in this process were observed as early as 12 hours after the onset of illumination. The changes included a pronounced increase in chlorophyll and carotenoid concentrations, accompanied by changes in the protein pattern and in the transformation of amyloplasts and leucoplasts to chloroplasts. The bleaching herbicides amitrole and norflurazon inhibited the synthesis of carotenoids in the illuminated potato microtubers. However, amitrole only delayed greening and an increase in chlorophyll and carotenoid levels became visible as late as four days after the onset of illumination, and the LHC II protein of the photosynthetic membrane was not detected before the seventh day of light exposure. Norflurazon, in contrast, acted as a stronger inhibitor, and microtuber tissues stayed yellowish throughout the experiment. The concentrations of both carotenoids and chlorophylls were very low in tissues treated with this herbicide. The LHC II protein could not be detected after a seven-day light exposure and the plastids were damaged, small in size, without normal thylakoids and with numerous plastoglobules.

Introduction

The action of many herbicides directed against chloroplast functions eventually results in the inhibition of carotenoid biosynthesis, in the destruction of chlorophylls, and in a dramatic alteration of thylakoid structures. The direct targets for such “bleaching” herbicides are enzymes involved in the biosynthesis of carotenoids present in the photosynthetic apparatus (Böger, 1996). One of these herbicides, amitrole, is known to interfere with the action of ζ -carotene desaturase, which catalyses the cyclization of lycopene, an intermediate in the biosynthesis of β -carotene (Barry and Pallett, 1990). Amitrole is also known to affect catalase activity (Feirabend and Kemmerich, 1983). Norflurazon, on the other hand, is known as an inhibitor of phytoene desaturase, which converts phytoene to ζ -carotene (Böger, 1996). Both “bleaching” herbicides inhibit the synthesis of β -carotene, which plays an important role in pho-

toprotection. In the absence of cyclic carotenoids, due to the action of excited singlet oxygen, chlorophyll will be oxidized and some proteins of the photosynthetic apparatus will be damaged (Siefermann-Harms, 1987). Chloroplast development is thus inhibited in bright light, but still proceeds to a limited extent if plants are grown at low light intensities. The use of herbicides, that interfere with the assembly of the photosynthetic membrane may thus provide additional information on the structural organization of this biomembrane.

Materials and Methods

Potato microtubers (*Solanum tuberosum* L. cv. Istra) were propagated from plants grown in tissue culture on MS medium (Murashige and Skoog, 1962), using the method of nodal segments (Wang and Hu, 1985). For the induction of microtubers we used MS medium supplemented with 8% sucrose, 0.5% agar, 1 mg/l thiamine-HCl and 5 mg/l benzylaminopurine, pH 6.0 (Wang and Hu, 1985). The material was then kept in the dark, at 18 °C, and 30 days old microtubers were used for experiments. They were placed on dry filter-paper, into covered glass Petri dishes, and moistened twice, during the first 12 hours, with a few drops of an

Abbreviations: LHC II protein, light-harvesting complex II protein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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aqueous solution of a final concentration of amitrole (1×10^{-3} M) or norflurazon (2×10^{-4} M). Control and treated potato microtubers were illuminated 16 hours per day with fluorescent light ($25 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) at room temperature. The material was examined after 12 hours, 2 days, 3 days, 4 days and 7 days.

The pigments were extracted with 100% acetone with the addition of potassium sulfate and measured spectrophotometrically. The pigments were quantitatively determined according to Lichenthaler (1987). For HPLC analyses, the pigments were isolated and examined according to the procedure described earlier (Muraja-Ljubičić *et al.*, 1998).

Membrane proteins were extracted as described previously (Lorković *et al.*, 1993). The protein content of the extracts was determined according to Bradford (1976). Proteins were then separated in 12% SDS polyacrylamide gels (Laemmli, 1970) and visualized by the silver stain technique. For Western blot analysis of LHC II protein, the procedure of Bollag and Edelstein (1991) was used. Gels were equilibrated and the proteins were transferred to a nitrocellulose membrane for 2 h. The membrane was washed and blocked overnight in 3% bovine serum albumin at 4 °C. Primary antibodies, raised in rats (a gift of Z. Lorković, Faculty of Science, Zagreb, Croatia) against the LHC II protein from potato were coupled with secondary goat-rat IgG antibodies. For the detection of the enzyme-coupled antibodies, an alkaline phosphatase assay was used (Muraja-Ljubičić *et al.*, 1998).

For electron microscopy small pieces of microtuber tissue were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.2), and after washing in buffer, were postfixed in 1% OsO_4 . After dehy-

dration, the material was embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 10A electron microscope.

Results and Discussion

The increase in chlorophyll and carotenoid concentrations, as observed by spectrophotometric measurements, was first detected in control microtuber tissue 12 hours after the onset of illumination, and within the next days their concentrations further increased (Table I). This process is much faster than that in ordinary potato tubers (Muraja-Fras *et al.*, 1994). In amitrole-treated microtubers the pigment content remained low in the first two days in the light, but started to increase after the third day. Norflurazon showed a much stronger influence on pigment biosynthesis. The concentrations of chlorophyll *a+b* and of colored carotenoids remained very low even after 4 days of illumination.

HPLC analyses showed results compatible with those observed by spectrophotometric measurements. The appearance of chlorophyll *a+b* in control microtubers was already detected after 12 hours of illumination and in the next days the increase in pigment content was well visible. In microtubers treated with amitrole and exposed to light a slight increase in chlorophyll content was detected after three days of illumination, while in those treated with norflurazon the overall content of both chlorophylls and colored carotenoids remained very low until the end of the experiment.

During the greening of control microtubers, characteristic membrane proteins of the photosynthetic apparatus soon appeared. After 12 hours of

Table I. Spectrophotometric measurement of chlorophyll *a+b* and colored carotenoids during greening of control potato microtubers and microtubers treated with amitrole and norflurazon.

Days in light	Chlorophyll <i>a+b</i> (ng g ⁻¹ fr. wt.)			Carotenoids (ng g ⁻¹ fr. wt.)		
	1.	2.	4.	1.	2.	4.
Control	3.39 ±0.57	8.62 ±1.07	25.35 ±1.92	3.68 ±0.28	7.37 ±0.78	15.41 ±1.19
Amitrole	3.01 ±0.20	3.86 ±0.32	14.37 ±0.30	3.04 ±0.37	4.96 ±0.12	9.94 ±1.10
Norflurazon	2.50 ±0.36	2.85 ±0.48	5.02 ±0.64	3.54 ±0.29	3.59 ±0.25	4.12 ±0.42

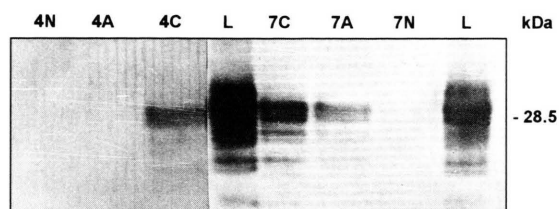


Fig. 1. Western blot analysis of LHC II protein from control potato microtubers and microtubers treated with amitrole and norflurazon after four (4C = control, 4A = amitrole treatment, 4N = norflurazon treatment) and seven days of light exposure (7C = control, 7A = amitrole treatment, 7N = norflurazon treatment); L = LHC II protein isolated from leaf.

illumination the LHC II protein was detected using Western blot analyses. After four days of light exposure the LHC II protein (ranging from 23–28.5 kDa) could be detected as a pronounced band (Fig. 1). Within this period of time, this protein was not detected in microtubers treated with

amitrole or norflurazon and exposed to light. After seven days of illumination, the LHC II protein was only observed in the amitrole samples, while in norflurazon samples no LHC II protein showed up, even after seven days of light exposure (Fig. 1).

After seven days of illumination, chloroamyloplasts and chloroplasts were clearly visible in control tissues. These plastids contained a well-developed thylakoid system and numerous ribosomes in their stroma (Fig. 2). Amitrole delayed plastid transformation for some days. In the almost white microtubers resulting from norflurazon treatment, the small plastids were practically without thylakoids and ribosomes, but with numerous large plastoglobules in the stroma (Fig. 3).

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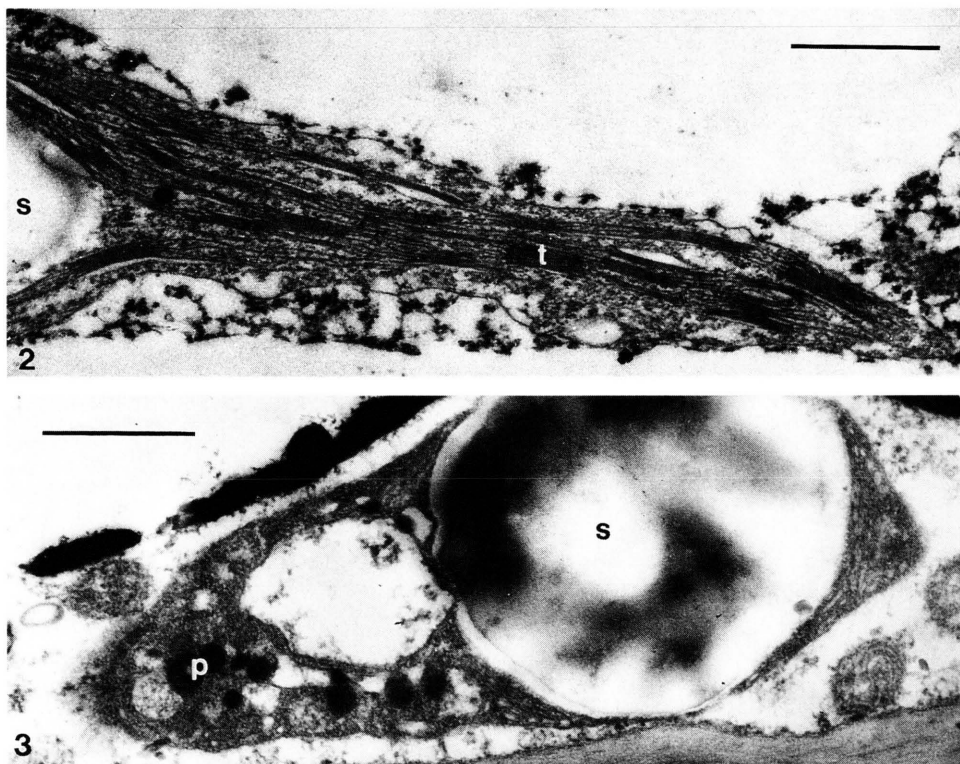


Fig. 2. Electron micrograph of a part of chloroamyloplast from potato microtuber after 7 days of light exposure; s = starch; t = thylakoids. Bar = 1 µm.

Fig. 3. Electron micrograph of a plastid from potato microtuber treated with norflurazon after 7 days of light exposure; p = plastoglobules; s = starch. Bar = 1 µm.

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