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DIFFERENTIAL RESPONSE TO THE HERBICIDAL ACTIVITY OF δ-AMINOLEVULINIC ACID IN PLANTS WITH HIGH AND LOW SOD ACTIVITY

YAIR ASKIRA, BARUCH RUBIN and HAIM D. RABINOWITCH

Department of Field and Vegetable Crops, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot, Israel

δ-aminolevulinic acid (ALA) is the obligatory precursor for tetrapyrroles and for chlorophylls in plants. Under illumination, these photosensitizers generate singlet oxygen, thus causing bleaching and death of treated tissues. We have examined whether superoxide is involved in the mode of action of ALA and whether SOD provides protection. Bean genotypes with similar carotenoid content but differing in SOD activity and cucumber seedlings were used throughout. Cucumber plants treated with 10 mM ethanolamine (EA) prior to ALA, had higher levels of chlorophyll fluorescence and lower values of electrolyte leakage than control. Bean cultivars with high SOD activity were considerably more tolerant to membrane damage caused by ALA than those with low SOD activity. SOD activity was greatly reduced in cucumber leaves treated with diethyldithiocarbamate (DDTC). Electrolyte leakage was markedly increased and chlorophyll fluorescence values were significantly lower in DDTC and ALA treated tissues as compared with those treated with ALA only. The results indicate that superoxide is involved in the toxicity caused by ALA and that, by breeding for high SOD activity, resistance to ALA can be achieved, thus allowing the use of ALA as a selective herbicide in the field.

KEY WORDS: Beans; cucumber; carotenoids; DDTC; ethanolamine; oxygen free radicals; singlet oxygen; superoxide; superoxide dismutase.

ABBREVIATIONS: ALA = δ-aminolevulinic acid; DDTC = diethyldithiocarbamate (sodium salt); EA = ethanolamine; EC = electrical conductivity; SOD = superoxide dismutase.

INTRODUCTION

δ-aminolevulinic acid (ALA) is the obligatory precursor for tetrapyrroles and for chlorophyll in plants. Plant tissues treated with ALA in the dark, synthesize and accummulate tetrapyrroles. The absorption of light by tetrapyrroles and by chlorophylls leads to their excitation to the triplet state which may directly damage biological molecules. Alternatively, these can interact with ground state oxygen, thus generating singlet oxygen. The latter may react with biological molecules, causing bleaching and death of the treated tissues.[1] Carotenoids may provide protection against damages caused by this oxygen species.[2] Rebeiz *et al.*[1] have proposed that ALA can be used as a natural herbicide.

Illuminated chloroplasts generate some other oxygen free radicals, e.g. superoxide, hydrogen peroxide and hydroxyl radical,[3] which may cause similar symptoms. Superoxide dismutase (SOD) provides protection against the potential toxicity of the superoxide radical. All three types of SODs have been found in plants, CuZnSOD occurs mainly within chloroplasts, and is the most abundant SOD in higher plants.[3]

Correspondence to: Dr. Haim D. Rabinowitch, Department of Field and Vegetable Crops, Faculty of Agriculture, PO Box 12 Rehovot, Israel 76 100.

This enzyme is deactivated by copper chelating agents[4] thus the toxic effects caused by superoxide are enhanced.

Chilling injuries in illuminated plant tissues are caused by both, singlet oxygen and superoxide.[5] Increase in the total phospholipid content in the leaves has been observed after low temperature hardening[6] and external application of amino alcohols, such as ethanolamine, enhanced cold tolerance in cucumber leaves.[7]

Cell lipids undergo peroxidation in the presence of oxygen free radicals. Consequently, chlorophyll fluorescence yield is decreasing[8] and electrolyte leakage is increasing,[9] both are used for determination of cell membrane integrity.[10]

In the present work, we have examined whether superoxide is involved in the mode of action of ALA and if SOD and phospholipids provide protection.

MATERIALS AND METHODS

Plant Material and General Experimental Procedures

Cucumber (cv. Beit-Alpha M.R.) and four bean genotypes were used throughout. Two disks 10 mm ϕ were sampled from expanded primary leaves (bean) or cotyledons (cucumber). These were immersed in 0.5 ml tap water (control) or in ALA solution. Multiwell tissue culture plates were used, allowing treatment to individual disks. The disks were shaken in ALA for 14 to 16 h in the dark, rinsed, and then shaken in DDTC for 1 h (when necessary). These were then moved to test tubes containing 10–15 ml distilled water, shaken for 1 h and then illuminated as detailed in the results.

Ethanolamine (EA) Treatment

Cucumber were germinated in vermiculite, seedlings at the cotyledonary stage were transferred into 50% Hoagland solution. At the first leaf stage, roots were thoroughly rinsed and the nutrient solution was exchanged for tap water (control) or to 10 mM EA solution. After 75 h, disks were sampled from the first leaf, treated with ALA as described above and tested for electrolyte leakage.

EC Measurements

Electrical conductivity (EC) measurements and calculations were according to Marsh and Davis[11] and to van de Dijk *et al.*,[9] with some modifications. The initial electrical conductivity using conductivity-meter model TH-72 (El Hama, Israel) was read. Light was turned on ($350\,\mu Em^{-2}sec^{-1}$), and 8 replications of 2 disks were sampled after 5, 15, 30 or 60 min. The disks were further shaken in the dark for 2 h and EC was measured. Following a 16 h storage at -20° C, the tissues were thawed and EC was read again, reflecting the entire electrolyte content of the tissues. Leakage was calculated from the readings obtained after illumination divided by the difference between the final and initial values.

ASSAYS

Chlorophyll Fluorescence was measured according to.[12] Each pair of treated and

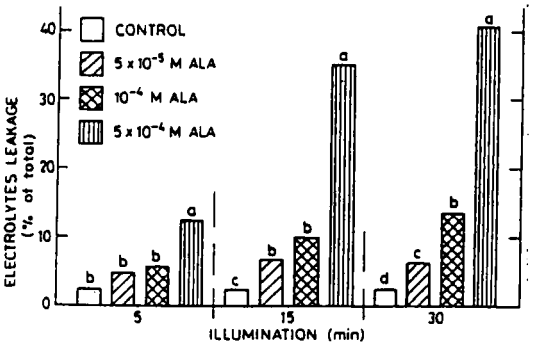

FIGURE 1 Effect of ALA concentration and light on electrolytes leakage from cucumber leaves.

Two disks 10 mm ϕ were sampled from each 7 to 10 d old seedlings, when cotyledons were fully expanded. Statistical comparisons were made between ALA treatments, for each illumination time separately. Values are means of 6 replications followed by the same letter are not significantly different at $p \leq 0.05$ by Duncan's multiple range test.

control disks, were sampled from one leaf. Two readings were taken, after 2 and 200 sec illumination, respectively. F_{max} is the maximal value of the fluorescence signal emitted from illuminated chlorophyll; F_v, the variable fluorescence is indicative of the reduction level of the primary acceptor of the photosynthetic apparatus (Q_A).[8]

Plastid Pigments conc was determined according to[13] and SOD was assayed photochemically.[14]

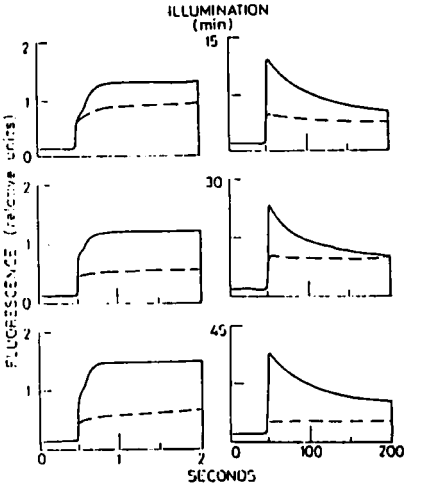

FIGURE 2 Effect of ALA at 5 × 10^{-4}M and illumination time on the chlorophyll fluorescence.

Cucumber leaf disks were sampled from opposite cotyledons, one treated with ALA, the other one served as control. At the end of the 15, 30 or 45 min illumination, light was turned off, and chlorophyll fluorescence measured. Two readings were taken, after 2 sec illumination (left column) and after 200 sec (right column). Dashed lines and unbroken lines represent fluorescence from ALA and from control disks, respectively.

RESULTS

Cucumber plants treated with 5×10^{-4}M ALA, showed a significant increase in electrolyte leakage already after five minutes of illumination, indicating that damage to the membranes occurs shortly after the light is turned on. With longer exposures to light, high values of electrical conductivity were measured also with ALA conc as low as 5×10^{-5}M (Figure 1).

Fifteen min illumination were required before damage to the photosynthetic system was noticed (Figure 2). F_{max} values markedly decreased with illumination time (left column), indicating damage to the primary electron acceptor of the photosynthetic apparatus. The decrease in the level of variable fluorescence during the long term

TABLE I

Effect of pretreatment with ethanolamine on the electrolyte leakage from ALA treated cucumber leaves. Cucumber seedlings were grown in liquid solution (50% Hoagland). At the first true leaf stage, these were transferred to 10mM EA solution for 75 h, afterwhich disks were sampled from the first leaf, and treated with ALA. Six replications per treatment

	EA (10mM)	Control
ALA (mM)	Electrolyte Leakage (%)	
0	5.2 ± 1.0	7.3 ± 1.0
0.1	9.0 ± 1.0	17.0 ± 1.0
2 × 0.1	15.2 ± 1.0	31.7 ± 3.0
3 × 0.1	26.0 ± 3.0	39.6 ± 4.0
5 × 0.1	31.4 ± 4.0	53.0 ± 6.0

measurements (right column), indicate a further damage to the electron transport system.[8]

Cucumber plants treated with EA prior to ALA, were less sensitive to the ALA treatment than control. Electrolyte leakage was significantly lower (Table I), and F_{max}

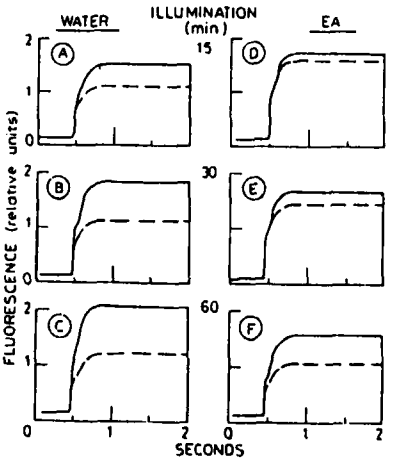

FIGURE 3 Effect of pretreatment with ethanolamine on the fluorescence signal from ALA treated cucumber leaves.

Experimental details, as in Table I, fluorescence measurements as in Figure 2.

TABLE II

SOD activity and chlorophyll to carotene ratio in four bean genotypes, and the electrical conductivity values of leaf disks treated with ALA. All disks were sampled from the first primary leaf, 8 replications per treatment

Bean genotype	SOD units/ Fresh wt. 1g	Specific activity	Chlorophyll /Carotene	EC% ALA (mM) 0	5 × 0.1
Jap-2	1963 a	89.5 a	29.4	6%	10% b
Jap-1	1763 a	84.7 a	30.2	5%	12% b
Nep-2	956 b	48.1 b	28.3	5%	50% a
Seaferer	879 b	41.4 b	28.6	6%	49% a
SE	178	9.6	2.4NS	1.25NS	6.5

values were higher (Figure 3). The protective effect of EA indicates that oxygen free radicals are involved in ALA toxicity.

Bean plants similar in both, carotenoid content and chlorophyll to carotenoid ratio, but varying in SOD activity showed a differential response to ALA. Those with high SOD, were markedly more tolerant to the injuries caused by ALA to the membranes, as shown by EC values (Table II).

Application of a copper chelator (DDTC) at 0.1 M following ALA treatment, accelerated electrolyte leakage from the treated tissues as compared with controls (Figure 4). DDTC is known to inhibit activity of CuZn-SOD.[3,4] Hence, the role of superoxide in the mode of ALA toxicity and the role of SOD is demonstrated.

DISCUSSION

Plants treated with δ-aminolevulinic acid biosynthesize and accumulate massive amounts of tetrapyrrole intermediates of the chlorophyll biosynthetic pathway, in the dark. When illuminated, these act as photodynamic sensitizers, thus generating singlet oxygen, which in turn causes bleaching and death to susceptible tissues.[1] Under illumination, these symptons occur in intact plants, in a matter of hours.[1] Our findings with leaf disks show, that green tissues suffer injuries in a matter of minutes. A four fold increase in EC was measured already after 5 min illumination, indicating severe damage to membrane integrity. In addition, a significant reduction in fluorescent

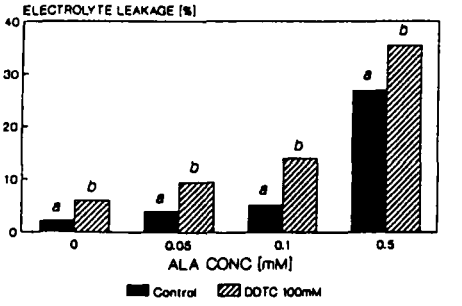

FIGURE 4 Effect of DDTC on the electrolyte leakage from ALA treated cucumber cotyledons.

signal clearly show that the photosynthetic apparatus is also impaired. The damage to the photosynthetic system is detectable within 15 min illumination. It is therefore concluded that damages to the internal and external cell membranes are the first step in the processes leading to the final symptoms of bleaching and death. The intensity of the damage is dependent on both, ALA concentration and the length of illumination period.

Addition of EA to cucumbers, caused an increase in the phospholipids total content and in their composition in the leaves. Also such treatment reduced chilling damages to these tissues.[7] Chilling damages are caused by photo-oxidative processes, and both singlet oxygen and superoxide are involved.[5] We have clearly shown that EA pretreated cucumber seedlings, had lower EC values and slower F_{max} quenching rates than those treated with ALA only. These results indicate that oxygen free radicals are involved in the damages caused by ALA.

In plants, superoxide is a commonly encountered intermediate of oxygen reduction and superoxide dismutases provide a defense against the phytotoxicity of this radical. In addition, all green cells contain carotenoid pigments which provide defense against photo-oxidative damages caused by singlet oxygen.[2] High SOD beans, tolerated higher ALA levels than bean plants with lower SOD activity. EC values were ca 10 and 50% of the total electrolytes, for the respective genotypes, indicating a significant more severe damage to the membranes of the latter plants. In addition, the inactivation of CuZnSOD by the copper chelating agent DDTC, significantly enhanced electrolyte leakage signifying once again the role of superoxide in the processes leading to injuries caused by ALA treatment.

CONCLUSIONS

The results indicate that superoxide is also involved in the toxicity caused by ALA and that by breeding for high SOD activity, a selective increase in tolerance to ALA can be achieved.

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