PHOTOSYNTHETICA 34 (2): 169-177, 1997

Short-term effects of aluminium at alkaline pH on the structure and function of the photosynthetic apparatus

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Abstract

A 24 h exposure of the salt-tolerant grass *Thinopyrum bessarabicum* (Savul. and Rayss) A. Love seedlings to 1 mM aluminium (Al) in nutrient solution at pH of 9.0 resulted in a significant reduction of the biomass. In control samples the mesophyll chloroplasts exhibited the usual lens shape with most grana arranged in straight or slightly curving lines, and only 6.5 % of the grana were out of order. In Al-treated plants the mesophyll chloroplasts displayed a slightly distorted shape and distended size with most grana arranged in bow-like lines, while in the central region of the organelle as many as 26.7 % of the grana were independent and out of order in relation to the long axis. The morphological changes in the chloroplast shape and grana arrangement were probably due to swelling and distension of the chloroplasts in consequence to the altered membrane permeability. The initial *in vivo* chlorophyll (Chl) fluorescence F_O, as well as the intermediate F_I and peak fluorescence F_P were increased under the Al stress: this indicated a destruction of photosystem (PS) 2 reaction centres and increased reduction of Q_A. The $(F_I-F_O)/(F_P-F_O)$ ratio exhibited a significant increase indicating higher proportion of PS2 centres unable to reduce Q_B. Changes in the chloroplast ultrastructure seemed to be the reason of photosynthetic electron transport inhibition. Yet all these changes in the photosynthetic performance and chloroplast ultrastructure were considered as indirect effects of Al treatment since Al concentration in the leaves was undetectable. Disturbances in the chloroplast ultrastructure could be caused by a reduced uptake and/or transport of other nutrients.

Additional key words: chlorophyll fluorescence; chloroplast ultrastructure; photosynthesis; photosystem 2; *Thinopyrum bessarabicum*.

Received 20 February 1996, *accepted* 25 November 1996.
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Abbreviations: Chl - chlorophyll; F_O, F_I, F_P, F_V - initial, intermediate, peak, and variable (F_P-F_O) fluorescence; PS2 - photosystem 2; Q_A, Q_B - primary and secondary quinone acceptors of PS2.
Acknowledgements: This research was made possible through research grants to M.M. provided by the Hellenic General Secretariat for Research and Technology, and the British Council in Athens.

Introduction

Aluminium (Al) has a toxic effect on the growth of agriculturally important plant species (Foy 1988, Taylor 1988). Therefore, the harmful effects of acid soils on the plant growth are closely related to Al toxicity (Bona *et al.* 1994). However, in the nature, Al is present not only in acid soils but also in alkaline ones. Soils may be polluted and become alkaline due to the alkaline fly ash or bauxite residue. The latter case is characterised by a high pH (9.0 to 12.0) and alkalinity, high concentrations of soluble sodium (Na) and aluminate [$Al(OH)_4^-$] (Fuller and Richardson 1986, Foy 1988, Eleftheriou *et al.* 1993).

The Al toxicity has been extensively studied in plant roots; less is known about its effects on leaves (Taylor 1988, Moustakas and Ouzounidou 1994, Moustakas *et al.* 1995). Phytotoxic effects of Al on the metabolism within leaf tissues have been demonstrated only in acid pH (Hampp and Schnabl 1975, Ohki 1986, Hoddinott and Richter 1987, Moustakas *et al.* 1995, Lorenc-Plucińska and Ziegler 1996).

The chloroplast thylakoid membrane contains a complex machinery responsible for the photosynthetic conversion of radiant energy to the chemically bound one. This fundamental energy conversion requires a coordinated participation of several different types of reactions such as the light-harvesting, charge separation, electron transport, proton translocation, and enzymatic catalysis. The Chl *a* fluorescence emitted from chloroplast thylakoid membranes reflects the primary processes of photosynthesis (Papageorgiou 1975, Lichtenthaler and Rinderle 1988). The yield of the Chl fluorescence is influenced in many ways by the state of the photosynthetic apparatus, and it indirectly senses the secondary changes induced at the membrane level in the course of photosynthetic reactions. Thus, the Chl *a* fluorescence changes have a potential for serving as a very early, non-destructive sign of stress injury (Lichtenthaler and Rinderle 1988, Bolhàr-Nordenkampf *et al.* 1989, Foyer *et al.* 1990, Krause and Weis 1991, Morales *et al.* 1991, Hák *et al.* 1993, Moustakas *et al.* 1994, Ouzounidou *et al.* 1995, Vidal *et al.* 1995).

Insight into the effect of Al toxicity on the photosynthetic energy conversion can be gained by measuring the Chl fluorescence. The wide use of Chl fluorescence measurements in stress research can be attributed to the advantages that the method provides: it is rapid, non-destructive, and can be used on small samples of the green plant material (Lichtenthaler and Rinderle 1988, Moustakas *et al.* 1993).

The purpose of this study was to examine whether a short-term (24 h) treatment by 1 mM Al had any effect on thylakoid photofunctioning, and if it concomitantly caused any ultrastructural changes in the chloroplasts of *T. bessarabicum* plants growing in alkaline (pH 9.0) nutrient solution.

Materials and methods

Plants: Seeds of the salt tolerant grass *T. bessarabicum* (Savul. and Rayss) A. Love (2x) were collected in the littoral zone at Agelochori, near Thessaloniki, Greece, and planted on nylon-meshed floats placed in a modified Hoagland nutrient solution

containing [μM]: KCl 50, H_3BO_3 25, $MnSO_4 \times H_2O$ 2, $ZnSO_4 \times 7H_2O$ 2, $(NH_4)_2Mo_7O_{24} \times 4H_2O$ 0.5, Fe-EDTA 20, $CuSO_4 \times 5H_2O$ 0.5. Nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur were supplied from KNO_3, $Ca(NO_3)_2$ $4H_2O$, $NH_4H_2PO_4$, and $MgSO_4 \times 7H_2O$ at concentrations [mM]: N 1.6, P 0.2, K 0.6, Ca 0.4, Mg 0.1, and S 0.1. The seedlings were grown in a phytotron programmed for a 14 h photoperiod with photosynthetic photon flux 150 ± 20 μmol m^{-2} s^{-1}, temperature 22 ± 1/18 ± 1 °C, and relative humidity 65 ± 2/75 ± 2 % day/night (Moustakas *et al.* 1992).

Aluminium treatment: Al was applied as a 1 mM $KAl(SO_4)_2 \times 12$ H_2O solution on the day 7 after germination. All nutrient solutions were adjusted to a pH of 9.0 by adding 5 mM Na_2CO_3. According to species computations, at a pH > 7.9 aluminate [$Al(OH)_4^-$] would constitute > 99 % of the mononuclear hydroxy-Al. However, the formation of a very toxic polynuclear Al_{13} species (Kinraide 1990) cannot be excluded. Thus, in our study we considered the total aluminium without implying a particular Al species. Plants were kept in the Al solution for 24 h. A concentration of 1 mM Al and a high pH were chosen to correspond with those of bauxite residue (Fuller and Richardson 1986, Eleftheriou *et al.* 1993). Other plants were grown in the modified Hoagland solution without Al for 24 h under the same conditions, and were treated as controls. The plants were harvested immediately after the 24 h treatment, and processed for the Al content measurement, electron microscopy observations, and the Chl fluorescence analysis.

Aluminium content: Oven-dried material of the second leaves of controls and 24 h Al-treated plants were wet digested in $HNO_3/HClO_4$ (4/1, v/v). The Al content was determined by the atomic absorption spectrophotometry (Moustakas *et al.* 1992).

Electron microscopy: Samples from the mid-region of the second leaf blades of the controls and Al-treated plants were processed for electron microscopy, as described by Eleftheriou *et al.* (1993). Briefly, the samples were fixed in 3 % v/v glutaraldehyde in 0.05 M cacodylate buffer for 3 h at room temperature, post-fixed in similarly buffered 1 % m/v OsO_4 for 3 h, dehydrated in a graded ethanol series, and embedded in Spurr's epoxy resin. Ultrathin sections, cut on a *Reichert-Jung Ultracut E* ultramicrotome using a diamond knife, were examined and photographed with a *Zeiss 9 S-2* electron microscope, after double staining with uranyl acetate and lead citrate.

Morphometric cytology: Chloroplast parameters, such as the organelle surface area and the grana surface fraction per chloroplast, were estimated using a point counting analysis technique, as described by Steer (1981) and Toth (1982). For this purpose, a transparent acetate sheet bearing intersecting lines spaced at 0.5 cm was used as a probe. The line intersections were treated as points for point fraction determinations. A total of 15 pictures of control and equal number of Al-treated samples were carefully selected with criterion to be mid-sectioned (judged from the plastid envelope appearance), and these were used for the morphometric analysis. The number of grana per chloroplast and the number of grana being out of order relative to the long axis were estimated by direct counting on the electron micrographs. The

angle of deviating grana relative to the long axis of the organelle was determined by a goniometer.

Chl fluorescence measurements *in vivo* were taken with a fluorometer (Plant Productivity Fluorometer model *SF-10, Richard Brancker Research*, Ottawa, Canada) on the upper surface of the second leaf after 24 h of Al treatment. The fluorescence signals were digitised, and values collected on a PC (Moustakas *et al.* 1993). The plants were dark adapted for at least 30 min at room temperature before the initial (F_O), intermediate (F_I), and peak (F_P) fluorescence parameters were measured at room temperature (Moustakas *et al.* 1994).

Statistics: The Chl fluorescence parameters and fresh mass of the above-ground parts of plants are means of 5 and 15 measurements, respectively. Values were statistically analysed by analysis of variance. Treatment mean differences were examined using the least significant difference at $p = 0.05$.

Results and discussion

Growth and aluminium content: The above-ground plant biomass was significantly lower (mean 21 %, $p = 0.001$) in plants grown at 1 mM Al compared to the controls [24 mg(fr.m.) plant^{-1}]. No Al was detected in the second leaf of the controls or Al-treated plants; the limit of detection was less than 1 μM. This suggests that no direct injury occurs in leaves as a result of exposing whole plants to Al for 24 h, possibly because toxic species of Al do not reach leaves in sufficient quantity to induce injury. As no Al was detected in the leaves, it was suggested that Al inhibited above-ground plant growth by reduced uptake and/or transport of nutrients (Moustakas *et al.* 1995, Strid 1996).

Table 1. Morphometric cytometry of chloroplast parameters. The differences between Al-treated and untreated plants are significant as determined by *t*-tests ($p < 0.005$). $n = 15$.

	Al [mM] 0	1
Surface area of chloroplasts [μm^2]	8.3±0.3	9.5±0.6
Surface area of grana/surface area of chloroplast [%]	26.9	27.5
Number of grana/chloroplast section	46.4±2.9	49.8±4.1
Number of grana out of order and allocation in angles relative to the long axis of the chloroplasts: 0-30°	1.2 (2.6 %)	4.9 (9.9 %)
30-60°	0.8 (1.7 %)	4.0 (8.0 %)
60-90°	1.0 (2.2 %)	4.4 (8.8 %)
total	3.0±1.3 (6.5 %)	13.3±1.8 (26.7 %)

Electron microscopy: The mesophyll chloroplasts of the second leaf of control plants in cross section had the usual lens shape with the internal membrane system oriented

Fig. 1. The mesophyll chloroplast of a control (*A*) or a 24 h Al-treated seedling (*B*) leaf of *Thinopyrum bessarabicum*. The great majority of grana is either arranged in ordered lines (*A*) or in bow-like lines with the exception of the central region where they are variously oriented (*B*). IS = intercellular space, V = vacuole.

along the longitudinal axis (Fig. 1). Grana were well developed with 2-12 thylakoids per granum, and were aligned in long, straight or slightly curving lines. Only 6.5 % of grana were out of order (Table 1). A few small osmiophilic globuli were dispersed throughout the moderately dense stroma.

Macroscopically, the appearance of leaves of 24 h Al-treated plants was normal. Ultrastructurally, most mesophyll chloroplasts displayed a slightly distorted shape (Fig. 2). The internal membrane system obtained an arch configuration with most grana arranged in bow-like lines. In the central region of the organelle, however, a high percentage of grana (26.7 %) appeared independent and oriented at several angles relative to the long axis (Table 1). This figure was statistically significantly different ($p < 0.005$) from that of the control samples. Other ultrastructural malformations of chloroplasts or of other cell components could not be detected, with the exception of a slight increase of the chloroplast surface area (Table 1).

The lack of extensive ultrastructural changes in leaf cells of Al-treated plants in the present study, possibly because toxic species of Al did not reach leaves in sufficient quantity to induce injury, was presumably due to the short-term exposure to Al and to the endodermis barrier (cf. Godbold *et al.* 1988). Root cells which come into direct contact with the Al solution suffer serious ultrastructural malformations, thus the primary site of Al toxicity are the peripheral cells of the root and the root cap both in acid (Bennet and Breen 1991, Ryan *et al.* 1993) and alkaline pH region (Eleftheriou *et al.* 1993). Isolated chloroplasts which come into direct contact with Al^{3+}-ions show a clear damage of the outer chloroplast membrane (Hampp and Schnabl 1975). In our case, since Al concentrations in the leaves were undetectable, it seemed that after a 24 h Al treatment the effects on the photosynthetic apparatus were indirect.

The morphological changes in chloroplast shape and grana arrangement are probably due to a swelling and distension of chloroplasts, presumably the result of alteration of membrane permeability (Anderson and Schaelling 1970). Disorientation of the internal membrane system and chloroplast swelling occur also in naturally senescing leaves (Hurkman 1979).

Chl fluorescence measurements: When the plants are exposed to environmental constraints, alteration of the functional state of the chloroplast thylakoid membranes usually results in changes in the characteristics of Chl fluorescence induction signals which can be used to quantify stress effects in leaves (Lichtenthaler and Rinderle 1988, Bolhàr-Nordenkampf *et al.* 1989, Krause and Weis 1991, Lichtenthaler 1992). Although the photosystems may not be the direct target of injury, fluorescence is sensitive to cellular disruptions including such caused by Al (Moustakas and Ouzounidou 1994). The strong relationship between Al tolerance determined *via* root tolerance index and fluorescence techniques supports the inference that rapid reductions in fluorescence reflect an actual tissue injury (Moustakas *et al.* 1993). On irradiation of the leaf, there was a fast rise in emitted fluorescence from an initial value of F_O to an intermediate stage F_I, and this was followed by a second, somewhat slower rise from F_I to a maximum value, F_P. The F_O level corresponded to constant emission of fluorescence, unaffected by the leaf photochemistry, and in a state of

maximum Q_A oxidation; the rise from F_O to F_I was produced by the photoreduction of Q_A, the F_I value reflecting the Q_A to Q_B equilibrium; the final increase to F_P (or F_m) corresponded to reduction of the plastoquinone pool; F_P thus denoted the maximum state of reduction reached by this pool.

Table 2. Effect of 24 h of Al-treatment on some chlorophyll fluorescence parameters of attached second leaves of *T. bessarabicum*. Values are means ± SE ($n = 5$). *Difference to control significant at $p < 0.05$.

Al-treatment [mM]	F_O	F_I	F_P	$(F_I - F_O)/(F_P - F_O)$
0	0.425±0.001	0.608±0.001	1.673±0.002	0.147±0.001
1	0.431±0.001*	0.627±0.003*	1.683±0.002*	0.157±0.001*

The initial Chl fluorescence F_O, as well as F_I and F_P, were significantly increased under Al stress (Table 2). An increase in F_O may occur if the PS2 reaction centres are damaged, or if the transfer of excitation energy from the antenna to the reaction centres is impeded (Bolhàr Nordenkampf *et al.* 1989).

The ratio F_I/F_P defined as $(F_I-F_O)/(F_P-F_O)$ is an excellent *in vivo* system for the determination of inhibition of PS2 photosynthetic activity (Shaw *et al.* 1985). The $(F_I-F_O)/(F_P-F_O)$ ratio measures the proportion of variable fluorescence leading to the F_I point in the Kautsky induction curve, and is an estimate of the proportion of PS2 centres unable to reduce Q_B (Morales *et al.* 1991, Ouzounidou 1993). Under Al stress, leaves of *T. bessarabicum* exhibited a significant ($p = 0.001$) increase (7 %) in the $(F_I-F_O)/(F_P-F_O)$ ratio (Table 2), suggesting a partial inhibition of photosynthetic electron transport at PS2 (de Prado *et al.* 1992, van Rensburg and Krüger 1993).

Changes in the chloroplast ultrastructure seem to be the reason of photosynthetic electron transport inhibition (Maksymiec *et al.* 1995). The Al stress at alkaline pH resulted in closure of the PS2 reaction centres and inhibition of primary photosynthetic reactions, mainly between Q_A and Q_B. Although the Al stress induced slight modifications of chloroplast ultrastructure, presumably due to the short-time exposure, they were indicative of a perturbation of the chloroplast structure, which could impair the attainment of optimal photosynthetic activities in *T. bessarabicum* leaves. However, all these changes in chloroplast ultrastructure and photosynthetic performance are considered as indirect effects of Al-treatment. Disturbances in the ultrastructure of chloroplasts may be caused by a reduced uptake and/or transport of other nutrients.

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