Effect of Evernic Acid on Structure of Spinach Chloroplasts

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ABSTRACT

Detached leaves of *Spinacia oleracea* were incubated with evernic acid, the main phenolic substance present in *Evernia prunastri* thalli. This lichen substance produced a decrease in the amount of total chlorophyll and chlorophyll a in treated spinach leaves. Chloroplast structure suffered a decrease in several parameters, i.e. chloroplast area, number of grana, granal width, number of thylakoids per granum and starch content. The submicroscopic structure of the chloroplast membranes revealed smaller particle diameters in several of the fracture faces in the evernic acid treated samples and even a decrease in the density of particles in the EF_s fracture face. The alterations observed may be related to changes in photosynthetic activity, probably by modification of both photosystem I and photosystem II activities.

Key words: Evernic acid, chloroplast structure, TEM, thylakoidal membrane, freeze-etching, chlorophyll content.

INTRODUCTION

Lichens as epiphytic autotrophs can have harmful effects on trees (Richardson, 1974). The destruction of corticolous lichens growing on fruit trees is a common practice in several parts of the world, in the belief that lichen-free trees are more robust (Hale, 1983). Epiphytic communities of lichens seem to produce an inhibition of tea shoot development (Asahina and Kurokawa, 1952) as well as a partial or complete defoliation of phorophyte in the wild holm-oak woods of Central Spain (Estevez *et al.*, 1982; Ascaso *et al.*, 1983).

Epiphytic lichens may penetrate the phorophyte tissues, reaching as far as the xylem (Ascaco *et al.*, 1980; Orus and Ascaso, 1982). Under natural conditions, the phenolic substances produced by lichens can potentially reach conducting tissues of the tree. The toxicity of such lichen phenolic substances has been demonstrated under laboratory conditions (Ramaut and Thonar, 1972*a, b*; Dauriac and Rondon, 1976). Moreover, phenolic substances isolated from the *Evernia prunastri* thallus and added to the incubation medium of detached leaves of *Quercus rotundifolia* inhibited photosystem II electron flow (Orus *et al.*, 1981) and affected the ultrastructure of the chloroplasts (Ascaso *et al.*, 1983).

Several authors have employed ultrathin sections and freeze-fracturing electron microscopy (Goodenough and Staehelin, 1971; Miller and Cushman, 1979; Miller, 1980; Popov *et al.*, 1981) to examine the supramolecular organization of the thylakoid membranes. Considering that chloroplast structure and function are intimately connected, our study concentrated on ultrastructural chloroplast changes in spinach leaves produced by evernic acid, which is the most abundant phenolic substance found in the *E. prunastri* thallus.

MATERIAL AND METHODS

Detached spinach leaves (obtained commercially) were incubated for one week with evernic acid (Sigma, 35.5 μM) in 1 mM NaHCO₃ pH 6.5. Samples incubated in the

bicarbonate solution without evernic acid were used as controls. In both cases detached leaves take up the solution through the petioles in the transpiration stream.

Chlorophyll determinations

Chloroplasts were isolated according to the method of Whatley and Arnon (1963). Chlorophyll determinations were made from 80 per cent acetone extracts of the material using Arnon's (1949) equations to calculate chlorophyll *a*, *b* and total chlorophyll concentration.

Electron microscopy

After treatment, leaf pieces of about 1 mm² were fixed in 3.25 per cent glutaraldehyde in 0.1 M Sorensen buffer, pH 7.1, washed in buffer and post-fixed in 1 per cent osmium tetroxide. Dehydration was done in graded series of ethanol. Samples were embedded in Spurr's medium (Spurr, 1969) and polymerized at 70 °C for 24 h. Sectioning was carried out with an Ultracut-E Reichert Ultratome. The sections were stained with lead citrate according to Reynolds (1963) for 6 min at 20 °C.

Freeze-etching

The submicroscopic structure of the chloroplast membranes was studied as described by Moor and Mühlethaler (1963). The chloroplasts were isolated from the spinach leaves after treatment. The chloroplast membranes were infiltrated with glycerol as a cryoprotectant, final concentration of 25–30 per cent for 1 h at 4 °C. Droplets of chloroplast membranes were frozen for 10 s with Freon 22, and stored in liquid nitrogen. The freeze-fracture was carried out at –110 °C and after the temperature reached –100 °C, samples were etched for 3 s. The replicas were cleaned in 50 per cent nitric acid plus potassium bichromate for 2 d and then in distilled water.

Ultrathin sections and freeze-etched replicas were examined with a Philips EM 300 electron microscope. Image analysis was carried out with a Mop-Videoplan (Kontron) semiautomatic image analyser.

RESULTS

Visual inspection showed that incubation with evernic acid induced the symptoms of chlorosis and desiccation in spinach leaves.

The total chlorophyll and chlorophyll *a* content as well as the ratio chlorophyll *a*/chlorophyll *b* decreased in the evernic acid treated samples (Table 1), whilst chlorophyll *b* content increased slightly.

The leaves of *S. oleracea* by TEM showed the typical arrangement of the cells of leaf

TABLE 1. *Photosynthetic characteristics of chloroplasts from control and evernic acid-treated spinach leaves*

	Total chlorophyll ($\mu\text{g g}^{-1}$ f.wt)	Chlorophyll <i>a</i> ($\mu\text{g g}^{-1}$ f.wt)	Chlorophyll <i>b</i> ($\mu\text{g g}^{-1}$ f.wt)	Chl <i>a</i> /Chl <i>b</i>
Control	1032.2 $\pm$ 47.0	614.3 $\pm$ 25.0	417.8 $\pm$ 22.0	1.47
Evernic acid	900.1 $\pm$ 47.2	431.2 $\pm$ 24.4	468.8 $\pm$ 25.0	0.92

Means $\pm$ s.d.

TABLE 2. Differences in the ultrastructure of chloroplasts from spinach leaves incubated in bicarbonate solution (control) and in evernic acid in bicarbonate solution (evernic acid)

Parameter	Control	Evernic acid
Area of chloroplasts (μm^2)	$6.58 \pm 1.50^*$	$3.71 \pm 1.61^*$
Average number of grana per chloroplast	$23.67 \pm 6.30^*$	$18.14 \pm 3.84^*$
Average granal width (nm)†	$117.50 \pm 53.4^*$	$100.90 \pm 51.6^*$
Average number of thylakoids per granum†	$5.96 \pm 2.96^{**}$	$5.30 \pm 2.87^{**}$
Percent starch content per chloroplast section (starch area as % of chloroplast area)	1.94	0.81

Means $\pm$ s.d., based on 100 median chloroplast sections.

Values of a specific feature are statistically different (*t*-test) at $*P < 0.01$; $**P < 0.05$.

† Based on 165 grana stacks.

tissue: upper epidermis, palisade and spongy parenchyma and lower epidermis. Since the cells of the spongy parenchyma are very near the vascular bundle, the present study focuses on the chloroplasts of these cells. The evernic acid-treated chloroplasts had a lower starch content, were smaller in area, contained fewer grana per chloroplast section and the grana stacks were narrower and contained fewer thylakoids (Table 2).

Freeze-fracturing splits membranes along their hydrophobic interior. The two complementary fracture faces are named according to the terminology of Branton *et al.* (1975), PF for the protoplasmic face and EF for the exoplasmic face. PF_u, PF_s and EF_u, EF_s are protoplasmic (PF) and exoplasmic (EF) fracture faces in unstacked (u) and stacked (s) membranes respectively. Figure 1 shows the fracture face of the chloroplast membranes of the control and the evernic acid-treated samples.

Histograms of particle size (Figs. 2 and 3) on thylakoid membrane fracture faces PF_s, PF_u, EF_s and EF_u, were obtained from photographs similar to Fig. 1. The relative frequency of particles smaller than 10 nm increased on the EF_s face of the chloroplasts treated with evernic acid whereas the particles with a diameter bigger than 14 nm had decreased. On the PF face (PF_s and PF_u), the relative frequency of particles smaller than 10 nm increased in the thylakoids of chloroplasts from leaves treated with evernic acid compared with the control leaves.

The average diameter of particles on each face was lower in the treated samples than in the control ones (Table 3), except for the EF_u face. Furthermore, the particle number on the EF_s face was lower in the evernic acid-treated spinach leaves (925 particles μm^{-2}) than in the control ones (1 211 particles μm^{-2}). No significant differences were found for this parameter on the other faces. The ratio of particle density on PF to that on EF, given as the coefficient of thylakoid membranes (K_{mt}), is also shown in Table 3.

DISCUSSION

The changes in the amount of total chlorophyll as well as chlorophyll *a* and *b* in the evernic acid-treated samples indicate that certain aspects of photosynthetic physiology may be subject to variation.

Since the majority of the photosystem II centres are located in the stacked regions of the membrane systems, whereas the centre of photosystem I is found in unstacked regions (Miller, 1980) in treated plants, the reduction in the area of chloroplasts, in the amount

FIG. 1. Freeze-fracture characteristics of chloroplast membranes of spinach plants. A, Control. B, Evernic acid-treated plant. EF_s, EF_u, PF_s and PF_u, exoplasmic (EF) and protoplasmic (PF) fracture faces in stacked (s) and unstacked (u) membranes.

FIG. 2. Particle size histograms made from measurements on the EF and PF fracture face (stacked) of membranes of chloroplasts isolated from spinach leaves. Based on 500 particles. A, Control, EFs, $\bar{x}$ = 13.54 nm, s = 2.86 nm; B, evernic acid EFs, $\bar{x}$ = 11.03 nm, s = 2.3 nm; C, Control, PFs, $\bar{x}$ = 9.93 nm, s = 1.97 nm; D, evernic acid, PFs $\bar{x}$ = 8.52 nm, s = 1.84.

FIG 3. Particles size histograms made from measurements on the EF and PF fracture face (unstacked) of membranes of chloroplasts isolated from spinach leaves. Based on 500 particles. A, Control, EFu, $\bar{x}=11.48$ nm, $s=2.76$ nm; B, evernic acid, EFu, $\bar{x}=11$ nm, $s=2.8$; C, Control, PFu, $\bar{x}=10.7$ nm, $s=2.19$; D, evernic acid, PFu, $\bar{x}=8.86$ nm, $s=2.17$ nm.

TABLE 3. Diameter and density of particles on the P and E complementary fracture faces of the chloroplast membranes for control and evernic-treated spinach plants

Face	Treatment	Particle size ($\bar{x}$) (nm)	Particle density ($\bar{x}$) (n μm^{-2})
EF _s	Control	13.54 $\pm$ 2.86*	1211 $\pm$ 270*
	Evernic acid	11.03 $\pm$ 2.30*	925 $\pm$ 271*
EF _u	Control	11.48 $\pm$ 2.76	737 $\pm$ 133
	Evernic acid	11.00 $\pm$ 2.80	846 $\pm$ 236
PF _s	Control	9.93 $\pm$ 1.97*	1306 $\pm$ 167
	Evernic acid	8.52 $\pm$ 1.84*	1405 $\pm$ 238
PF _u	Control	10.70 $\pm$ 2.19*	1358 $\pm$ 223
	Evernic acid	8.86 $\pm$ 2.17*	1393 $\pm$ 208
K _{mt} PF _s /EF _s	Control	1.07	
	Evernic acid	1.51	
K _{mt} PF _u /EF _u	Control	1.84	
	Evernic acid	1.64	

Means $\pm$ s.d.

Values of a specific feature are statistically different (*t*-test) at **P* < 0.01.

of grana per chloroplast and, even more important, the decrease in stacking of thylakoidal membranes suggests the existence of alterations in the activity of the photosystems.

Since stacking is not the only condition for the PS II activity (Park and Nolan, 1974; Arntzen and Briantais, 1975), it was necessary also to observe the particles of the

thylakoidal membranes revealed by freeze-fracturing. Arntzen *et al.* (1969) previously suggested that the largest EF particles might be associated with Photosystem II and the smallest PF particles with Photosystem I. In a more recent study with *Pisum sativum* mutants, Popov *et al.* (1981) demonstrated a direct connection between changes in the particles and impairment of PS I and PS II activity. These authors found large differences in particle concentrations and relative population area (per μm^2) and also in particle size distribution, between mutant chloroplast membranes and the wild-type.

The changes observed by the above authors with regard to the distribution of particle size in mutants with a PS II deficiency basically consist of an increase in the frequency of the classes of smaller particle diameter and are similar to the ones found in the present study after incubating the spinach leaves in evernic acid.

Very recently, moreover, Semenova and Ladigyn (1984) suggested a model according to which the PS II in association with the LH complex forms the 14–22 nm particles of the EF face, with the 10–14 nm particles found on the EF fracture face, belonging to the PS II complex, while the PS I complex is formed by 10–14 nm particles on the PF fracture face. Therefore, in the case of the thylakoid membranes incubated in evernic acid, both the changes in the distribution of particle size, mentioned above, and the smaller average values of the particles on the EF_s, PF_s, and PF_u faces must be understood as a change integrity of the particles commonly associated with both photosystems. Furthermore, the lower particle density on the EF_s face in the treated chloroplasts can also be interpreted indicating reduced PS II content (Miller and Cushman, 1979).

The K_{mt} for stacked membrane regions with normal PS II has a low value in comparison with unstacked membrane regions which also have normal PS I (Popov *et al.*, 1981). An increase of K_{mt} correlates with a loss in PS II activity (Popov *et al.*, 1977). We have observed in our study that the K_{mt} for the stacked regions is higher for the evernic acid-treated samples (1.51) than for the controls (1.07), suggesting a loss of photosystem II activity. The changes in the membrane particles on both fracture faces of thylakoid membranes in the evernic acid-treated spinach plants indicate an important potential effect of evernic acid on photosynthesis. Since previous studies (Ascaso *et al.*, 1980; Orus and Ascaso, 1982) showed that epiphytic lichen hyphae reach the xylem it is probable that the phenolic substances from the epiphytic thallus (e.g. evernic acid) reach the xylem and are translocated to the phorophyte leaves, producing similar changes in their chloroplasts.

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