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Short note

Plasmalemma redox systems and membrane depolarization in leaf cells of *Olea europaea* L.

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Abbreviations: B.S.: basal solution; DCMU: (3,4dichlorophenyl)-1,1dimethylurea.(Sigma); Em: membrane potential; MES: 2-N-morpholinoethane-sulphonic acid (Sigma); *p*HMB: p-hydroxymercuribenzoic acid (Sigma); PM: plasma membrane; TRIS: 2-amino-2-hydroxymethyl-1,3-propanediol (Fluka).

Abstract: An initial electrophysiological study was carried out on PM redox systems in leaf cells of *Olea europaea* L. Perfusion of leaf segments with two membrane nonpermeating electron acceptors, ferricyanide [$K_3Fe(CN)_6$] and the ferric chelate Fe(III)NaEDTA, led to membrane depolarization of about 8 mV in the light. Dark and DCMU markedly reduced the depolarization effect of the electron acceptors. Intact leaves could not be used in the trials since the epidermis was impermeable to both the electron acceptors. The obtained results suggest that in olive leaves there are two different PM redox systems at work ("standard" and "turbo") or two states of the same redox system ("turbo").

1. Introduction

It is known that plants possess, at the plasmalemma level, enzymatic systems able to transfer electrons from a cytosolic donor to an apoplastic acceptor (Bienfait, 1985; Crane *et al.*, 1985; Medina *et al.*, 1997; Bérczi and Møller, 2000). These systems, called plasma membrane (PM) redox systems, are believed to be involved in important physiologic processes such as ion uptake, iron reduction, blue light responses, defence against pathogen attack through the generation of reactive oxygen species, cell wall synthesis, and signal transduction (Døring *et al.*, 1990; Døring and Lüthje, 1996; Medina *et al.*, 1997; Bérczi and Møller, 2000; Larbi *et al.*, 2001).

It has been suggested that two types of oxidoreductases exist in root cells: the "standard" oxidoreductase able to reduce iron (III) of ferricyanide [$K_3Fe(CN)_6$] but not that of the synthetic ferric chelates such as Fe(III)NaEDTA; and "turbo" oxidoreductase able, on the other hand, to reduce iron (III) of both ferricyanide and ferric chelates (Bienfait, 1988). Instead, in cells of the mesophyll, the presence of "standard" oxidoreductase has been noted (Dharmawardhane *et al.*, 1987; Neufeld and Bown, 1987; Askerlund *et al.*, 1991), but information on the "turbo" type is much less consistent (de la Guardia and Alcántara, 1996). This latter redox system plays an important role in iron absorption by cells of the mesophyll. In fact within the roots, the Fe (II) absorbed from the soil is reoxidized to Fe (III) and translocated to the leaves as citrate. As it cannot be assimilated by the cells in the (III) form, it is again reduced to Fe (II) by the putative "turbo" reductase (Tiffin, 1970; Böttger *et al.*, 1991).

Among the various studies produced until now on the subject, knowledge of PM redox systems in tissues of fruit tree species is almost completely lacking (Brancadoro *et al.*, 1995). Keeping in mind that many of the processes which appear to be involved in these systems may have important practical consequences, it was considered worthy of interest to undertake an initial study on leaves from olive trees, a species of great economic importance in the Mediterranean basin.

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2. Materials and Methods

General

There are two recognized methods for investigating PM redox systems. One is based on isolation and study of the plasma membranes. The other examines the effects exerted by different redox agents interacting with plasmalemma redox systems at the level of single cells or tissues (Rubinstein *et al.*, 1984; Barr *et al.*, 1985; Crane *et al.*, 1985; Møller and Lin, 1986; Ivankina and Novak, 1988). In the present work, the latter approach was employed, using as electron acceptors ferricyanide and the synthetic ferric chelate Fe(III)NaEDTA, both impermeable through plasmalemma. When a solution containing these electron acceptors diffuses in the apoplast, Fe (III) is reduced to Fe (II) by the PM redox systems and the phenomenon is accompanied by more or less notable depolarization of the plasmalemma (Marré *et al.*, 1988). Indeed, it is these variations in membrane potential that are examined in this initial series of trials.

Plant material

The study was conducted on olive leaves (cv. Leccino) removed from 10-year-old plants trained in vase and uniform for growth and productivity. Sampling was carried out in May on one-year shoots which were between 25 and 35 cm long and positioned in the outer portions of the crown.

Electrophysiological tests were carried out, initially, on intact leaves, and then, on 2x10 mm leaf segments, excised each one by a new blade scalpel handle.

Chemicals

Before beginning the electrophysiological experiments, the leaf segments and the intact leaves were incubated in basal solution (B.S.) in the light for 2 hr. During this period, the solution was renewed twice and constantly aerated. The salts present in the basal solution were only in sulphate form. The presence of Cl^- anions was avoided since their entry by symport with H^+ can influence cytoplasmic pH (Ullrich and Novacky, 1992; Bellando *et al.*, 1995). For the electrophysiological experiments, the components for the various treatments were added to the B.S. (Table 1). Except where indicated otherwise, the concentrations of K^+ and Na^+ in the B.S. and the treatments were adjusted to equal values. DCMU, a specific inhibitor of CO_2 fixation and O_2 evolution (Marré *et al.*, 1989), was employed in 0.05% ethanol, starting from a stock solution of 0.5 mM. Controls, in trials with DCMU, also contained the same percentage of ethanol as the treatments. *p*HMB, a strong inhibitor of Fe (III) reduction (de la Guardia and Alcántara, 1996), was dissolved in 0.1 M KOH.

Electrophysiology

Membrane potential (Em) was measured according to standard electrophysiological technique as previously adopted in olive leaves (Rinaldelli, 2000; 2004), with some modification. In brief, leaf segments or intact leaves, after incubation in B.S., were horizontally mounted in a 3 ml Plexiglass chamber secured to a microscope stage. Continuously aerated B.S. or treatments (Table 1) were permitted to perfuse through the chamber at a flow rate of 10 ml min^{-1}. They reached the tissue by force of gravity, each through its own adductor channel controlled by a manual valve. An integrated circuit temperature sensor (590 KH) placed inside the chamber recorded the temperature of the solution. Heating or cooling of the solution was obtained by way of a Peltier-effect heat pump located along the solution conduit before the Plexiglass chamber and was electronically controlled.

Table 1 - Composition of the solutions employed in the experiments

Basal solution (B.S.)	Treatments [z]
$CaSO_4$ 0.5 mM	$K_3Fe(CN)_6$ 0.5 mM
K_2SO_4 0.75 mM	Fe(III)NaEDTA [y] 0.5 mM
MES 5.0 mM	DCMU 10 µM
Na_2SO_4 [x] 0.5 mM	*p*HMB 0.5 mM

[z] All the solutions were prepared starting from the basal solution. All solutions were adjusted to pH 6.0 with TRIS.

[y] The solution with Fe(III)NaEDTA also contained K_2SO_4 (0.75 mM).

[x] Present only when treatment was carried out with Fe(III)NaEDTA.

The measuring electrodes used were micropipettes (tip diameter <1 µm) obtained from single-barrelled borosilicate capillaries (W.P.I., USA) by way of a vertical homebuilt puller. The micropipettes and the reference electrode were filled with KCl 3M. The electrodes were connected, by Ag/AgCl wires, to a high input impedance electrometer (10^{14} W). The output signals from the electrometer, before being transmitted to a chart recorder, were passed through a low-pass filter (10 Hz) in order to eliminate possible disturbance. Insertion of the microelectrodes took place under 250 X magnification, perpendicular to the tissue, in the central zone of the mesophyll by way of a motorized micromanipulator. For the intact leaves, instead, insertion into cells of the mesophyll took place by way of stomata. Treatments started after Em stabilized for 5 min.

Except where indicated otherwise, preincubation and electrophysiological tests were carried out at +20°C (± 0.4) in the light (30 W/m^2).

Statistical

The number of repetitions for each experiment is reported in the captions for each figure.

3. Results and Discussion

Treatments on leaf segments and intact leaves

From this first series of experiments, different behaviour by leaf segments and intact leaves emerged for treatments with $K_3Fe(CN)_6$ and Fe(III)NaEDTA.

By varying the potassium content in the B.S. which bathed the tissue before treatment, it was possible to highlight the following responses. When the B.S. and treatment were both adjusted to 1.5 mM of K^+ (0.75 mM di K_2SO_4), there was depolarization only in the leaf segments (Fig. 1, A). Instead when the B.S. lacked potassium, there was depolarization in both leaf segments and intact leaves. In the latter case, however, the amount of depolarization was less (Fig. 1, B). Finally, when treatment was carried out with only K_2SO_4 (0.75 mM), there was depolarization of equal amount in both leaf segments and intact leaves (Fig. 1, C). The amount of this depolarization was nearly the same as that obtained in intact leaves when the B.S. was devoid of potassium and the effect on the membrane was determined only by the K^+ of the ferricyanide (Fig. 1, B).

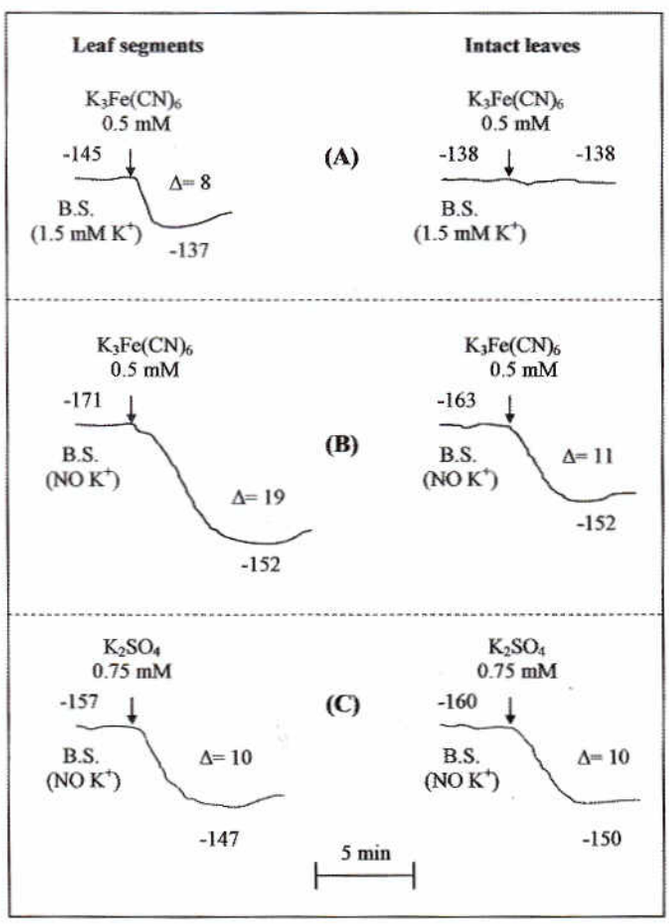

Fig. 1 - Effects of $K_3Fe(CN)_6$ (A, B) and K_2SO_4 (C) on membrane potential in leaf segments (left) and intact leaves (right). Measurements are expressed in mV. In (A) B.S. and treatments have the same potassium concentrations. In (B) potassium is present only in the treatments. Traces are representative of five equivalent measurements. ± SE was less than 5% of the mean.

Based on the results obtained, it can be assumed that among leaf segments and intact leaves a different permeability to complexed iron exists but not to potassium. On the basis of this assumption, it is possible to explain the different responses obtained with the two types of leaf tissues studied.

As shown in figure 1 (A), the K^+ concentrations in the B.S. and treatment are the same (1.5 mM). Depolarization, noted only in leaf segments, can be attributed solely to the ferric complex that is unable to pass through the epidermal barrier of intact leaves.

Figure 1 (B) shows potassium as being present only in the treatment, while in the B.S. it was absent. In leaf segments, where ferricyanide diffuses in the apoplast, depolarization is caused by both the potassium and iron which is reduced. The degree of the phenomenon is, in fact, greater than that in the previous case (A). In intact leaves, instead, there is a degree of depolarization which is equal to that measured in the treatment when potassium was supplied as the sole permeable ion.

Leaf segments and intact leaves, as shown in figure 1 (C) for the same potassium concentration, demonstrate an approximately equal depolarization. This confirms that potassium is equally permeable in both systems.

Analogous results to those reported in figure 1 were obtained when Fe(III)NaEDTA was employed (data not shown).

Based on these results, subsequent electrophysiological trials with the two ferric complexes were carried out on leaf segments only.

Role of light, dark and inhibitors

Figure 2 reports the Em effects exerted by $K_3Fe(CN)_6$ and Fe(III)NaEDTA in light, dark and in the presence of inhibitors, such as DCMU and *p*HMB.

Under light conditions the two electron acceptors demonstrated approximately the same efficacy, depolarizing with a ΔEm of 7-8 mV (Fig. 2 A), while under dark condition this effect was extremely low (Fig. 2 B). The role of light can be explained by the greater production, by chloroplasts, of triose phosphates. These, exported to the cytosol, give rise to NAD(P)H production which acts as an electron donor for the PM redox system (Bienfait, 1988; de la Guardia and Alcántara, 1996). This mechanism has also been noted in the reduction of nitrates (Huppe and Turpin, 1994). It follows that under illumination there is greater reduction of $K_3Fe(CN)_6$ and Fe(III)NaEDTA, as compared to dark conditions, and that this effect leads to greater membrane depolarization. The low depolarization noted in the dark for the two ferric complexes can be attributed to reduction phenomena caused by electron donors produced by glycolysis, pentose phosphate pathway, ascorbate or glutathione (Huppe and Turpin, 1994).

Pretreatment with inhibitors under illumination diminished the extent of depolarization caused by both

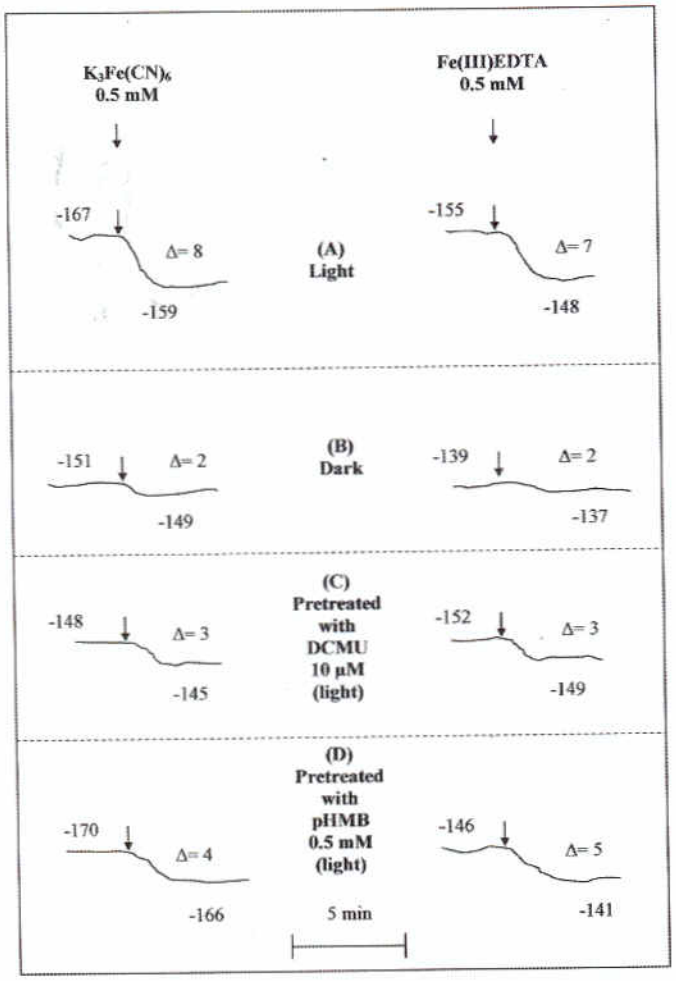

Fig. 2 - Leaf segments: effects of $K_3Fe(CN)_6$ and Fe(III)NaEDTA on membrane potential as influenced by light (A), dark (B), and inhibitors (C, D). Traces are representative of five equivalent measurements. ± SE was less than 5% of the mean.

electron acceptors [$K_3Fe(CN)_6$ and Fe(III)NaEDTA]. In particular, depolarization was slightly lower with DCMU (Fig. 2 C, D). Within the context of the obtained results, inhibition of photosynthesis by DCMU confirms the evidences arising from the trials under light and dark conditions (Fig. 2 A, B). Thus, light plays a primary role in the functioning of PM redox systems. In this regard, further support can be found in literature (Dharmawardhane *et al.*, 1987; Neufeld and Bown, 1987; Ivankina and Novak, 1988; de la Guardia and Alcántara, 1996).

Also when pretreating with *p*HMB, an inhibitor of PM redox systems that reacts with –SH groups of proteins (Serrano *et al.*, 1994; de la Guardia and Alcántara, 1996), the depolarization caused by the two electron acceptors was lower (Fig. 2, D) than the control (Fig. 2 A).

In conclusion, the effects exerted by the electron acceptors on membrane potential have highlighted the activity of two PM redox systems, termed "standard" and "turbo", or of two states of the same redox system ("turbo"). The operation of the latter system suggests that, also in olive, absorption of iron by the cells of the mesophyll may take place after its reduction to the (II) form.

In addition, from the studies reported here, it can be said that the activity of the PM redox systems is strongly dependent on light.

These initial experiments indicate that also in olive leaves a transplasmalemma electron transport is regulated by PM redox systems. Knowledge of the functioning of this mechanism may yield important information regarding a broad range of physiological processes.

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