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Short- and long-term effect of sulphite on sucrose transport in grapevine (*Vitis vinifera* L.) leaves. An electrophysiological study

E. Rinaldelli, R. Bandinelli, M. Pagano
Dipartimento di Scienze delle Produzioni Vegetali, del Suolo e dell'Ambiente Agroforestale, Sezione Coltivazioni Arboree, Laboratorio di Elettrofisiologia, Università degli Studi di Firenze, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy.

Key words: membrane potential, sucrose transport, sulphite, sulphur dioxide, *Vitis vinifera* L.

Abstract: Sucrose is the main carbohydrate translocated in grapevine and its transport may be restricted or inhibited by a number of factors such as the pollutant sulphur dioxide. The present study investigated, for the first time in grapevine, the effects of sulphite on membrane electrical response of sucrose transport in the mesophyll cells. Co-transport of sucrose across the membrane is linked to the free energy in a electrochemical proton gradient. Without the pollutant, electrophysiological traces displayed a metabolic-dependent sucrose electrical response in which an initial depolarization was followed by complete repolarization. In the presence of sulphite, instead, there were different trends depending on time of contact with the tissue of the pollutant. In the short-term, a slower repolarization was observed and in the long-term (after 6 and 12 h) the extent of depolarization (Δ mV) was also reduced. Transmembrane electrical potentials, measured in the presence of sulphite, became significantly ($P < 0.01$) more positive with increasing time of incubation of the tissue. On the whole, electrophysiological results highlight a direct or indirect effect of the pollutant on the activity of proton pump H^+/ATP ase. Since carbohydrate translocation has a central role in the balance between source and sinks in the plant, the results of the research suggest that sulphite can modify the above balance with negative implications for the export of carbohydrate from the leaves.

1. Introduction

The potential impact of atmospheric deposition of pollutants such as sulphur dioxide on vegetation has been the subject of many papers (Tingey and Olszyk, 1985; Darral, 1989; Mesanza *et al*., 1996; Johnson *et al.*, 1999). Plant injury caused by air pollution is most common near large cities and industrial enterprises, and damage in isolated areas occurs when pollutants are spread over long distances by wind currents (Cicek, 2003). Some studies have also begun to surface from developing countries (Hassan *et al*., 1995; Wahid *et al*., 1995), where yield losses of cereals and legumes have been attributed to both SO_2 and ozone. Exposure to SO_2 has been reported to decrease or inhibit photosynthesis (Silvius *et al*., 1975; Ziegler, 1975; Carlson, 1983; Rao *et al*., 1983; Katainen *et al*., 1987; Kropff, 1987; Sheu, 1994; Lorenc-Plucińska, 1998; Ranieri *et al*., 1999) and pollen tube growth (Karnosky and Stairs, 1974; Varshney and Varshney, 1981).

Even if the SO_2 sensitivity of grapevine is poorly documented (Ishikawa, 1972; Weinstein, 1984; Garcia-Huidobro *et al*., 2001), the species is considered sensitive to chronic SO_2 exposure, and adverse effects on growth or yield were presumed possible in the field (Daines, 1968; Fujiwara, 1970). Development of black and brown lesions was observed especially on leaves (Cicek, 2003); shoot growth reduction and, in some cases, leaf abscission were reported (Shertz *et al*., 1980). The interaction of SO_2 with ozone is evident. The combination of the two gases led to an increase in oxidant stipple in leaves (Forsline *et al*., 1983). In other species this interaction has not occurred (Kreess *et al*., 1986), suggesting the role of the genotype in resistance to the pollutant. Interaction SO_2/carbon black was also suggested by Ionescu *et al*. (1971) in the industrialized Copsa Mica zone of Romania, where the damaging effects of sulphur-containing effluents were enhanced by the presence of carbon black. Fujiwara (1970) found, with increasing SO_2 concentration, that leaf abscission, in cv. Fredonia, began earlier and progressed at a greater rate. Effects of sulphur dioxide were also found on the stomatal apparate of *Vitis labrusca* L. cv. Ives, where SO_2 induced both stomatal closure and a higher stomatal resistance (Rosen *et al.,* 1978).

Unlike other species (Lorenc-Plucińska and Ziegler, 1989; Maurousset and Bonnemain, 1990), there is not, for the vine, specific knowledge on the effects of SO_2 on

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sugar transport in leaves. In *Phaseolus vulgaris* and *Ricinus communis* SO_2 inhibits assimilate translocation and this inhibition seems to depend on damage to the sucrose transport system or proton pump (Noyes, 1980; Teh and Swanson, 1982; Lorenc-Plucińska and Ziegler, 1987 a, b, 1988). According to a more recent and detailed study on purified plasma membrane vesicles of *Ricinus communis*, the decreased uptake of sucrose may be attributed to a dissipation in the transmembrane pH gradient (Russell *et al.,* 1999).

Sucrose transport in leaves is a secondary active transport (co-transport) in which solute (sucrose) translocation across the membrane is linked to the free energy available in a proton electrochemical potential difference (Bush, 1993). It is widely known in the leaves of many plants (Giaquinta, 1979; Delrot, 1981; Delrot and Bonnemain, 1981; Huber and Moreland, 1981), and also in the vine is assumed to be so (Mullins *et al.,* 1992). Since sucrose transport involves the entry of protons into the cell, it gives rise to changes in transmembrane potential. Thus electrophysiological techniques are very useful for following, in real time, the transport of solutes. Regarding the effect of sulphite on sucrose transport, the problem has been studied mainly on a biochemical level, while there is little information on the electrophysiological aspects. To our knowledge, only one paper (Maurousset and Bonnemain, 1990) reports some information about these aspects, however it does not include combined electrophysiological tests with sucrose and sulphite. Therefore we felt it was interesting to investigate, for the first time in grapevine, how sucrose membrane electrical response was influenced by SO_2 supplied as sulphite.

2. Materials and Methods

Plant material

Electrophysiological tests were carried out on leaves of *Vitis vinifera* L., cv. Sangiovese clone SS-F9-A5-48, removed from two-year-old plants grown in a container and grafted on rootstock 420 A. Preliminary tests were performed on whole leaves, while the subsequent tests were on leaf segments (3 x 7 mm), cut with a scalpel under B.S. solution.

Chemicals

For electrophysiological experiments, the substance to be tested has to be dissolved in the solution bathing the tissue (treatment solution). Sulphur dioxide is highly soluble in water but, being gaseous, it is not easy to dose the exact amount to be dissolved. Therefore, in this research, Na_2SO_3 was employed, which at pH 5.5, is found mainly in the form of bisulfite ion (HSO^{3-}). Previous research conducted on isolated chloroplasts of *Spinacia oleracea* L. showed that SO_2 and bisulphite ions, at the same equimolecular concentration, have a parallel mode of action in the inhibition of photosynthetic oxygen evolution (Silvius *et al*., 1975). Moreover the effects of SO_2 on chloroplast enzyme systems were studied by means of hydration products of bisulphite and sulphite (Ziegler, 1975).

The components of the various treatments were added to the basal solution (B.S.) for the electrophysiological experiments (Table 1). In all treatments in which Na_2SO_3 alone was employed, even the B.S. contained the same amount of sodium supplied as Na_2SO_4. Carbonyl cyanide m-chloro phenyl hydrazone (CCCP), a protonophore and uncoupler of oxidative phosphorilation (Marrè *et al*., 1973) was used in 0.05% ethanol, starting from a stock solution of 0.5 mol m^{-3}. Controls, in the different trials, also contained the same percentage of ethanol in the treatments. 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 (Bellando *et al*., 1995).

Table 1 - Composition of the basal solution (B.S.) and concentration of the components in the treatments

Basal solution		Treatment components*	
K_2SO_4	2.5	sucrose	20.0
$CaSO_4$	0.5	Na_2SO_3	1.0
MES	5.0	CCCP	10^{-2}

All solutions were adjusted to pH 5.5 by way of TRIS or dilute H_2SO_4 when Na_2SO_3 was present. MES: 2-N-morpholinoethane-sulphonic acid (Sigma); CCCP: Carbonyl cyanide m-chlorophenylhydrazone (Sigma); TRIS: 2-amino-2-hydroxymethyl-1,3-propanediol (Fluka). *Concentrations are in mol m^{-3}.

Electrophysiology

Before beginning the electrophysiological experiments, whole leaves and/or leaf segments were incubated in basal solution in the light for 2 h (short-term). During this period, the solution was renewed twice and constantly aerated. Subsequent tests were performed after incubation of leaf segments in sulphite for 6 and 12 h (long-term). Even in this case the solution was constantly aerated and renewed every hour. Membrane potential (Em) was measured according to standard electrophysiological technique as previously adopted (Rinaldelli and Bandinelli, 1999; Rinaldelli, 2005) with some modification. In brief, whole leaves and/or leaf segments were mounted on a Poly(methyl methacrylate) chamber secured to a microscope stage. The chamber for whole leaves was slightly inclined and wide enough to accommodate a grapevine leaf, while the one for the segments was small (3 ml) and placed horizontally. Continuously aerated B.S. or treatments were permitted to perfuse through the chamber at a flow rate of 10 ml min^{-1}. They reached the tissue by gravity, each through its own adductor channel controlled by a manual valve. In the case of whole leaf, only the stomatal area around the insertion point and the petiole were perfused. A small glass thermometer placed inside the chamber allowed verification of 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 Poly(methyl methacrylate) chamber; it was electronically controlled.

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 500 mol m^{-3} KCl. The electrodes were connected, by Ag/AgCl wires, to a high input impedence electrometer (AD 549, 10^{15} Ω). 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 noise. The insertion of microelectrodes into the cells of the central part of the mesophyll took place under two different magnifications. In the case of whole leaves the electrode passed through a stoma and a magnification of 500x was necessary, obtained with a 50x long working distance objective (Mitutoyo, Japan). In the case of leaf segments, instead, a magnification of 250x, obtained with a 25 x long working distance objective (WPI, USA), was sufficient. The insertion of the microelectrodes was, in both cases, at an angle of about 45°, and took place by way of a very stable, manual, three-axis, homebuilt micromanipulator.

Treatments started after Em stabilized for 5 min. All experiments were performed under Faraday cage. The complete electrophysiological set-up is presented in figure 1. Except where indicated otherwise, preincubation and electrophysiological tests were carried out at +22°C (± 0.5) in the light (30 watt/m^2).

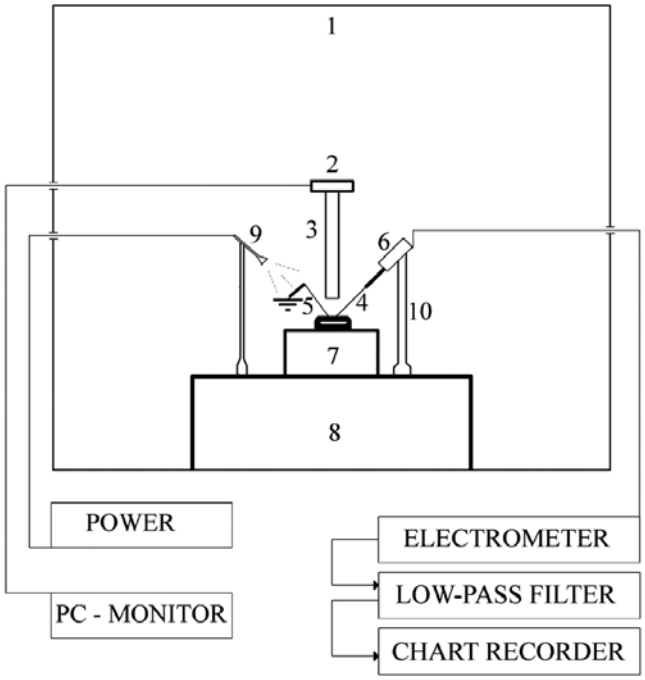

Fig. 1 Schematic electrophysiological set-up for membrane potential measurements. 1. Faraday cage. 2. Digital cameras. 3. Microscope tube. 4. Measuring microelectrode. 5. Reference electrode. 6. Probe. 7. Microscope stage. 8. Vibration-free table. 9. Lamp. 10. Micromanipulator.

Statistical analysis

The data relating to transmembrane potentials, measured at different times, in BS and sulphite, were subjected to statistical analysis of variance (ANOVA). Comparisons were carried out using Duncan's test. Statistical significance of differences were accepted when P <0.01. In the electrophysiological traces, depolarizations (Δ mV) are presented as mean ± SE.

3. Results

Preliminary experiments on whole leaves and leaf segments

Since the cuticle is often a barrier to organic molecules (Rinaldelli and Bandinelli, 1999; Rinaldelli, 2005), before working on a tissue it is necessary to verify its permeability to the solutions that will be employed. Thus, preliminary tests were performed on whole leaves and leaf segments. They showed that both were permeable to sulphite (Fig. 2, a, c) but not to sucrose. In whole leaves, in fact, sucrose depolarization was almost one-quarter that in leaf segments (Fig. 2, b, d). Based on this evaluation, all subsequent tests were performed on leaf segments.

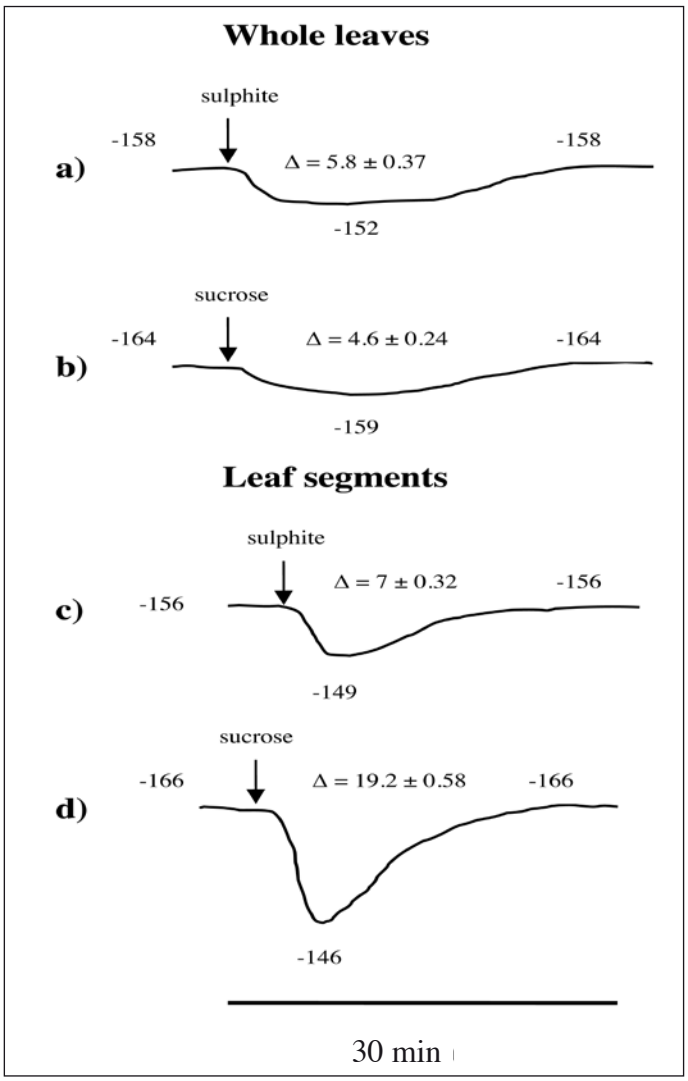

Fig. 2 - Effects of treatments with sucrose and sulphite on whole leaves and leaf segments. All measurements were carried out in light. Numbers preceded by - are negative mV. Electrophysiological traces are representative of five equivalent measurements. Δ is the depolarization (mV) as the mean of five measurements ± SE.

Sucrose transport and its metabolic support

As co-transport of sucrose in grapevine leaves was assumed (Mullins *et al*., 1992) but not electrophysiologically investigated, initial tests were carried out to verify this assumption. In the light, in the presence of the uncoupler CCCP, sucrose depolarization was less than half, compared to the same test without the inhibitor (Fig. 3 a, b). Subsequent repolarization was also very slow. In the dark, instead, sucrose depolarization was lower than in the light, and it was completely abolished by the uncoupler (Fig. 3 c, d). The different extent of depolarization, in light and dark, reflects metabolic support to the operation sucrose/H^+ symport. As discussed later, CCCP only inhibited oxidative phosphorylation and had incomplete or no effect on photophosphorylation.

At 5°C sucrose depolarization was nearly annulled (Fig. 3 e) and a similar response occurred when the pH of the treatment solution was brought to 8.0 (Fig. 3 f).

Short- and long-term experiments under sulphite

In the short-term tests, sucrose depolarization, in the light, was about 20 mV (Fig. 3 b) whereas in the dark it was much less (Fig. 3 c). Also, in the presence of sulphite, differences in extent of depolarization, in light and dark, remained approximately the same as previous tests without pollutant, but a slower repolarization was observed (Fig. 4 a, b). Based on this evidence, in the short-term sulphite affected only in part the membrane electrical response of sucrose transport.

Figure 5 shows the electrical responses to sucrose after leaf segments were preincubated in B.S. (control) or sulphite, for 6 and 12 h. Since short- and long-term treatments in the dark showed the same trends, only the first are reported.

Sulphite already reduced the sucrose depolarization after 6 h of incubation (Fig. 5, b), but the result was more evident after 12 h where the extent of depolarization de-

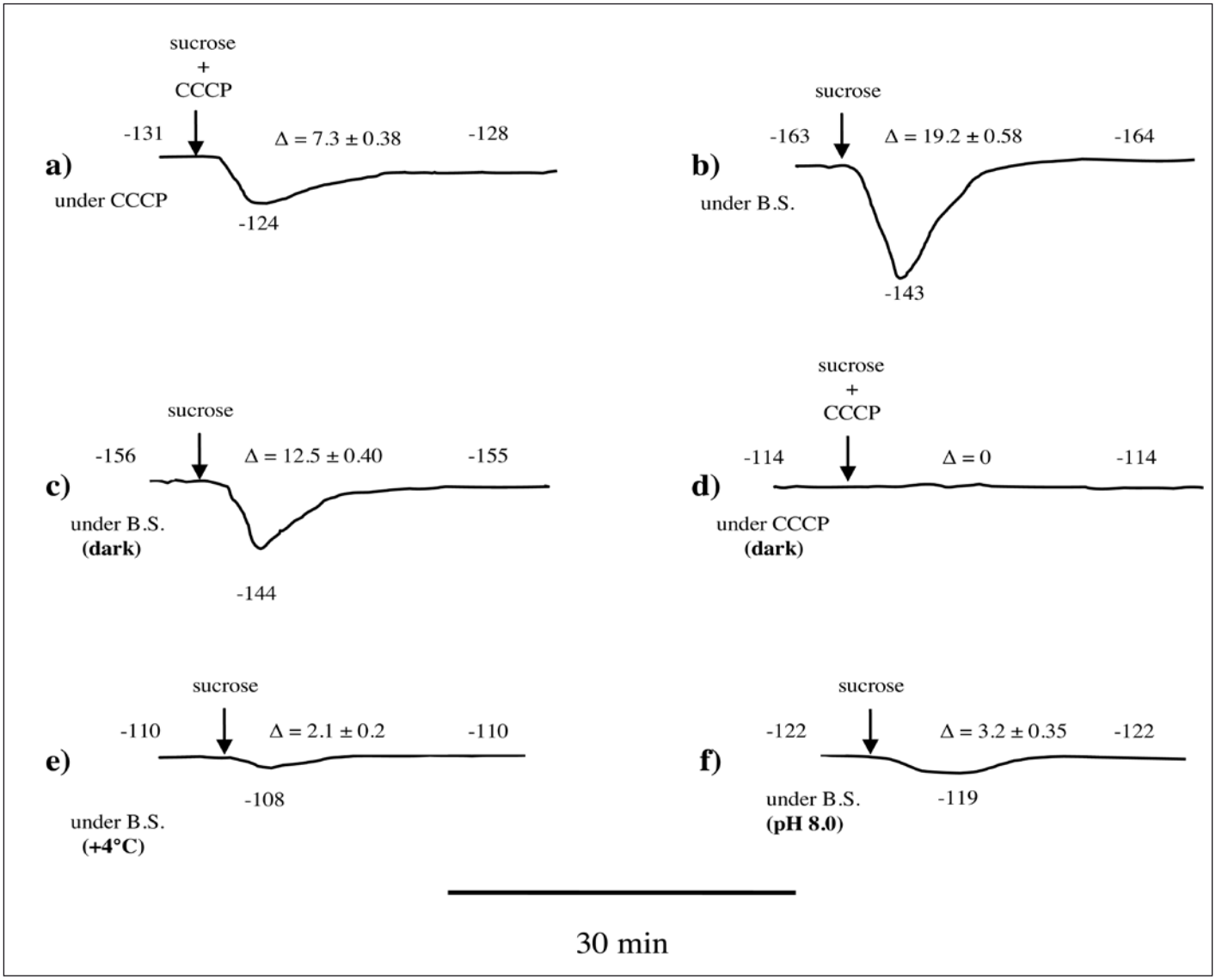

Fig. 3 - Effects of chemical and physical treatments to verify energetic support of sucrose transport. Except where indicated otherwise, measurements were carried out in light. Solution at pH 8.0 was buffered with 2 mol m^{-3} HEPES [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid] -TRIS. Numbers preceded by - are negative mV. Electrophysiological traces are representative of five equivalent measurements. Δ is the depolarization (mV) as the mean of five measurements ± SE

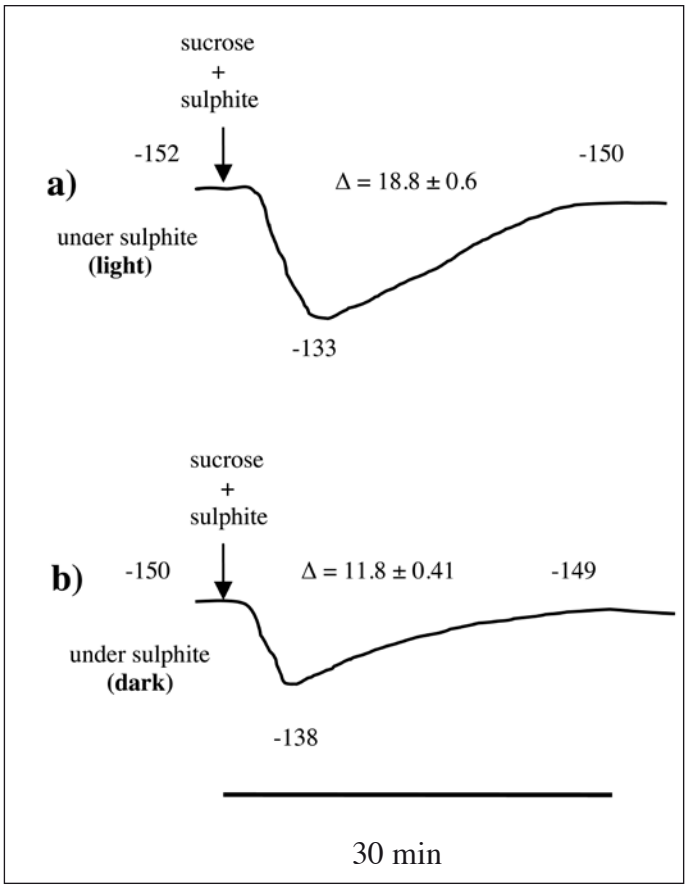

Fig. 4 - Short-term treatments in light and dark. Numbers preceded by - are negative mV. Electrophysiological traces are representative of five equivalent measurements. Δ is the depolarization (mV) as the mean of five measurements ± SE.

creased by almost half as compared to the control (Fig. 5d). Also the repolarization, after 6 h, and even more after 12 h, was strongly slowed. It should be noted that even with increasing incubation time there is a progressive decrease in the extent of depolarization. Also the transmembrane potentials decreased (they became more positive) with increasing incubation time (Table 2). These effects were evident under both, B.S. and sulphite. Under sulphite, however, they were more marked.

Table 2 - Membrane potentials (mV) recorded at different times of incubation, in B.S. and sulphite

	Membrane potentials	
Incubation time (h)	B.S. (no sulphite)	1 mol m^{-3} Na_2SO_3
0	-160.2 ± 2.61 a	-153.0 ± 2.05 b
6	-152.2 ± 2.04 b	-139.7 ± 2.45 d
12	-149.0 ± 2.44 c	-125.1 ± 2.33 e

Each value is the mean ± SD of ten experiments.

Different letters denote statistically significant differences between membrane potentials (P<0.01).

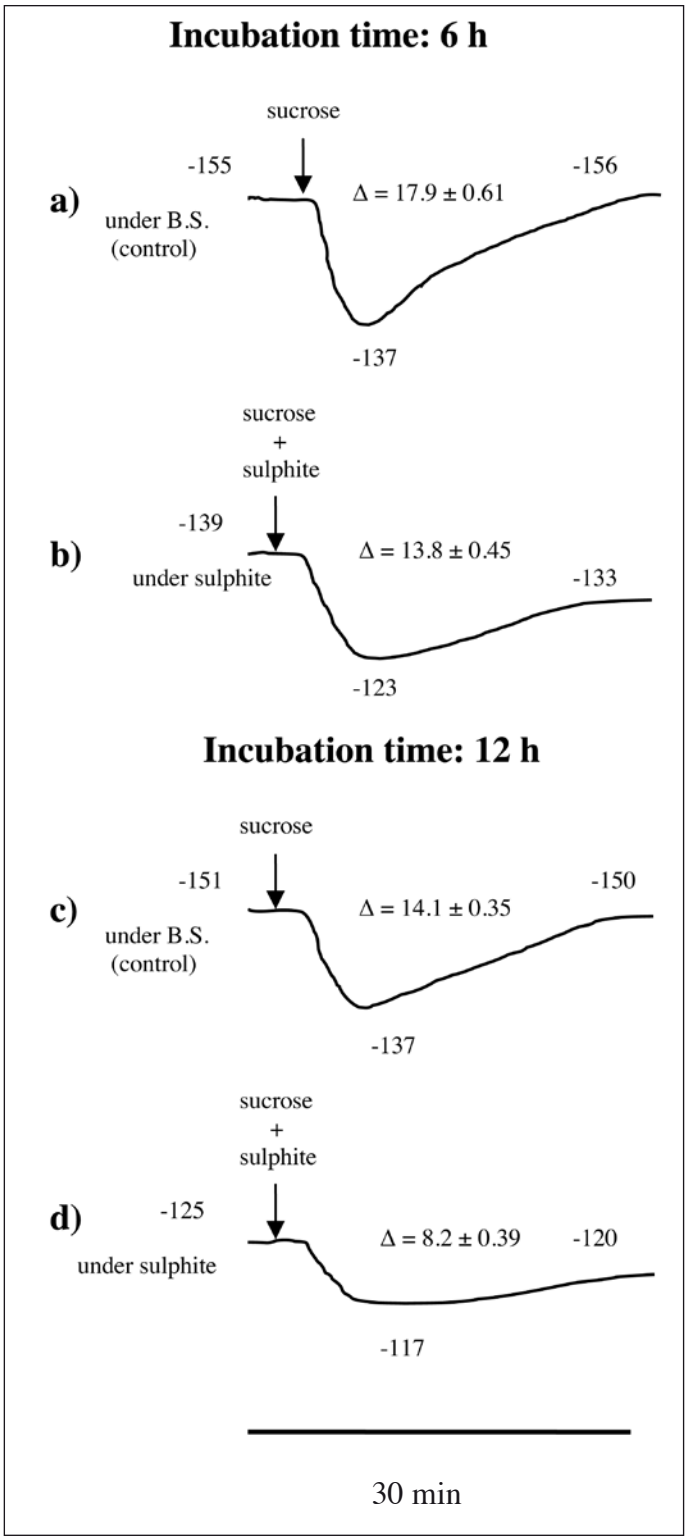

Fig. 5 - Long-term treatments, after 6 h and 12 h of incubation in B.S. and/or sulphite. All measurements were carried out in light. Numbers preceded by - are negative mV. Electrophysiological traces are representative of five equivalent measurements. Δ is the depolarization (mV) as the mean of five measurements ± SE.

4. Discussion and Conclusions

Energetic support to sucrose transport

CCCP is an uncoupler of oxidative phosphorilation which, in agreement with the chemiosmotic hypothesis (Mitchell, 1961), dissipates the electrochemical gradient

of H^+ ions preventing ATP synthesis. Consequently, active transport systems (primary and also, indirectly, secondary), lacking energy, are deactived.

Under CCCP, membrane electrical response of sucrose showed, in light and dark, two different results (Fig. 3 a, d). This finding suggests that the metabolic support of the operation of sucrose/H^+ symport comes from both respiration and photosynthesis. In dark, since there is no photosynthesis, ATP production is only of respiratory origin and it is blocked by the uncoupler CCCP. In light, instead, some energetic support appears because CCCP does not inhibit, or only partially inhibits, the photophosphorylation. This may be the case, taking into account the variable effects of CCCP on photophosphorylation as a function of leaf greening (Oelze-Karow and Butler, 1971; Butler *et al*., 1972) or light intensity (Saha *et al*., 1970; Prins *et al*., 1980).

Since chemical inhibitors may simultaneously affect many different processes of cells (Khalilov *et al*., 2002), we also tested the effect of low temperature. Treatment with sucrose at 5°C nearly led to the annulment of the depolarization. This suggests a metabolic dependence of sucrose transport on the activity of a plasmalemma ATP-driven proton pump. At 5°C, since the metabolic energetic support is highly limited (Mengel and Shubert, 1985; Rinaldelli and Bandinelli, 1999; Rinaldelli, 2000; 2004), the activity of the pump is inhibited.

The use of sucrose solution buffered at pH 8.0 strongly reduced depolarization. Under these conditions, the lack of protons in the apoplast would explain the annulment of H^+ co-transport. This response has also been observed for nitrate (Ullrich and Novacky, 1981; McClure *et al*., 1990) and urea co-transports (Rinaldelli, 2004).

All these results are considered sufficient to support a metabolic and pH-dependent sucrose co-transport.

Membrane electrical responses under sulphite

Sucrose is the main carbohydrate translocated in grapevine. From the leaves, where it is produced, it moves to the various sinks according to the specific needs of the plant (Coombe and McCarthy, 2000; Hunter and Ruffner, 2001). During the annual vegetative and reproductive cycle, it plays a preeminent role in regulation of the carbon/nitrogen ratio (Rodriguez-Lovelle and Gaudillère, 2002).

Sucrose, being a non-polar molecule, does not have direct effects on membrane potential when it enters the cell, but only indirect, because its transport is related to the flow of protons that move along an electrochemical gradient. This co-transport results in an initial depolarization observed under both B.S. and sulphite (Figs. 2 b, d; 3, 4, 5). Under sulphite, it has to be excluded that the depolarization could be due to Na^+ or the accompanying anion, since sucrose and perfusing solutions contained an equal amount of Na_2SO_3.

Repolarization of the membrane, which sometimes exceeded the starting level (overshoot), is proposed to depend on stimulation of the H^+-ATPase, caused by either changes in the cytoplasmic pH or the membrane potential itself. The H^+-ATPase has a pH optimum at 6.6, i.e. well below the physiological pH of the cell cytoplasm (usually around 7.2-7.5). Thus, whenever protons start accumulating in the cytoplasm, the activity of the pump increases to remove excess protons from the cell (Michelet and Boutry, 1995). This response is comparable, as cause and effect, to the one caused by permeant weak acids (Marrè *et al*., 1983; Rinaldelli and Bandinelli, 1999).

Sulphite alone, at the concentration used, induced a slight depolarization followed by partial or complete recovery. This is visible in both whole leaves (Fig. 2 a) and leaf segments (Fig. 2 c). Maurosset and Bonnemain (1990) found that the depolarization by sulphite, does not depend on the concentration used. Effect of the pollutant on the membrane would be, initially, that of "exciting agent". Instead, the repolarization would depend, according to the same authors, on the concentration of sulphite in the treatment. In our study we tested sulphite concentrations below and above 1 mol m^{-3} (data not shown) finding, however, less marked differences compared to the aforementioned authors. However the comparison is difficult because the study of Maurosset and Bonnemain (1990) did not include our same electrophysiological tests. Their tests, in addition, were conducted on a different species (*Vicia faba* L.) and this may also affect the reported results. When the treatment with sucrose was under sulphite, however, repolarization slowed and decreased gradually moving from short- to long-term. This is the first and most important effect of sulphite on sucrose transport. Since repolarization is dependent on proton pump activity, the direct or indirect effect of sulphite should be placed at this level.

In the long-term, under B.S. and sulphite, sucrose depolarization was lower (Fig. 5) and the transmembrane electropotentials became significantly more positive (Table 2). Since these effects are evident even in B.S., they should be attributed, at least in part, to phenomena of senescence which start when leaves are detached or segments are cut (Thimann *et al*., 1977; Gepstein, 1982; Malik, 1982). However, since the variations under sulphite were greater, there is clear evidence, in addition to senescence, of a direct or indirect effect of pollutant on proton pump activity. Proton pumps, in fact, are considered the primary motors that build up transmembrane electrochemical proton gradients (Felle, 2001).

The complex of electrophysiological tests performed in this research highlights a clear membrane electrical response of sucrose transport to sulphite. Two points stand out clearly enough: a) in grapevine leaves sucrose membrane electrical response is supported by metabolic energy; b) sulphite alters the above response by acting on the proton pump and/or energetic metabolism, from which the pump draws energy to extrude H^+ ions outside the cell. An effect of sulphite on respiration and photosynthesis cannot be excluded, especially in the long-term. The link may be there because both slowed repolarization and more positive transmembrane potentials under sulphite reflect a partial inhibition of the proton pump, whose activity is supported by respiratory and photosynthetic ATP.

The results obtained constitute an interesting acquisition in grapevine physiology, but it should be taken in account that conditions for the SO_2 effect, in the vineyard ecosystem, may differ from those adopted in the laboratory due to the high number of cultural, climatic and pedological variables.

Acknowledgements

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