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Stress effects of flumioxazin herbicide on grapevine (*Vitis vinifera* L.) grown in vitro

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Abstract Among the herbicides used in vineyards, the pre-emergence soil-applied flumioxazin (FMX) is a recently synthesized molecule that inhibits chlorophyll biosynthesis in weed species. The aim of this work was to characterize the effects of FMX on non-target grapevine (*Vitis vinifera* L. cv. Chardonnay) plantlets grown in vitro. FMX treatment (from 1 to 100 μM) represented a stress, as revealed by measurement of several parameters. Stem and leaves underwent dehydration and a decrease in both water- and osmotic-potential. Treated plantlets exhibited concomitant accumulation of soluble carbohydrates in all tissues and of free proline in stems and leaves. Moreover, FMX caused lipid peroxidation and electrolyte leakage in leaf tissues. These results indicate that the herbicide FMX is toxic for grapevine grown in vitro. In addition to inhibiting protoporphyrinogen IX oxidase, it causes water stress and membrane alteration in tissues and, as a consequence, generates the accumulation of carbohydrates and free proline.

Keywords Carbohydrates · Herbicide · Proline · *Vitis vinifera* L. · Water status

Abbreviations *FMX:* Flumioxazin · *MDA:* Malondialdehyde · *PROTOX:* Protoporphyrinogen IX oxidase · *TBARS:* Thiobarbituric acid reactive substances · *TFAA:* Total free amino acids · *TSS:* Total soluble sugars

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Introduction

Herbicides are commonly used in modern agriculture to improve crop quality and yield. Although they contribute to crop protection, their persistence in foods or the environment has raised public concern. Moreover, herbicide treatments may have secondary adverse effects on non-target plants. Many authors have reported that non-selective herbicides such as 2,4-D, glyphosate, chlorsulfuron or trichloroacetate may cause severe damage to crops by inducing leaf necrosis, decrease in germination, accumulation of reactive oxygen species or reduction of net photosynthesis (Bhatti et al. 1997, 1998; Radetski et al. 2000). In addition, some herbicides can affect nitrogen metabolism; a decline in leaf and root nitrate reductase activity has been reported in the presence of metribuzin (Lewosz et al. 1998). Accordingly, Scarponi et al. (2001) showed a decrease in the amino acid and protein content of maize treated with imazamox, as well as an increase in soluble sugars. On the other hand, Romera et al. (1990) reported that several photosynthesis-inhibiting herbicides caused the accumulation of protein and non-protein nitrogen content in olive trees.

Among the herbicides used in vineyards, the pre-emergence flumioxazin (FMX) is a recently synthesized molecule of the *N*-phenylphthalimide family, which inhibits protoporphyrinogen IX oxidase (PROTOX; E.C. 1.3.3.4), an enzyme involved in chlorophyll biosynthesis. The commercial product (Pledge) displays a strong efficacy towards grasses and broadleaf weeds. Previous studies have been reported on plants treated with diphenyl ethers, another family of PROTOX inhibitors, indicating a reduction of chlorophyll and carotenoid levels and subsequent lipid peroxidation (Wakabayashi and Böger 1995; Moreland 1999). Nevertheless, the few references concerning FMX do not give any information about the potential effects of this herbicide on non-target plants (Tomlin 2000).

Although it is a pre-emergence soil-applied herbicide, FMX may have dramatic consequences for grapevine if brought into contact with vine roots or leaves through

violent rainfalls or storms. In a preliminary attempt to evaluate the impact of the FMX herbicide on in vitro grapevine growth and photosynthetic performance, we recently reported the results of FMX treatments on in vitro-grown grapevine (Saladin et al. 2003). In that study, FMX impaired some functions of grapevine leaves when applied to the culture medium. As expected from its mode of action, FMX caused a decrease in chlorophyll content in the leaf tissues and a dramatic reduction in biomass production, photosynthetic gas exchange and leaf carotenoid content. These data strongly suggest that FMX causes a stress to the whole plant.

In order to further characterize the effects of FMX on grapevine, we evaluated plant reaction to the herbicide through the analysis of some physiological parameters known to be involved in stress responses, such as the water status of the plant, the index of membrane injury and the level of compatible solutes. The model chosen for this study was plantlets grown in vitro with adapted concentrations of the herbicide. Despite the physiological differences from plants grown in vineyards, the use of in vitro-grown plants provides new insights into the real mode of action of this herbicide (Saladin et al. 2003).

Materials and methods

Plant material, treatment and sampling

Microcuttings of *V. vinifera* L. cv. Chardonnay were grown in glass vials (150 mm ×25 mm diameter) containing 10 ml Martin medium (Martin et al. 1987) at 26°C under a 75 μmol m^{-2} s^{-1} photon flux density, a 16 h light/8 h dark photoperiod and 80–85% relative humidity. After 6 weeks, plantlets had 4.5 cm stems, six leaves, and roots were 7.5 mg dry weight. They were transferred onto new Martin medium solidified by 6% purified agar and containing 0 (control), 1, 10 or 100 μM FMX herbicide that had been directly dissolved in the medium before autoclaving. The concentration of the FMX herbicide was adapted to the survival of the plantlets according to Saladin et al. (2003). Plantlets were sampled before the transfer (day 0) and after 7, 14 and 21 days of treatment. Whatever the tested parameter, the sampling was performed at the same time of day so that circadian physiological fluctuations (i.e., photosynthesis) did not interfere with the determination of various soluble compounds.

Water status

The water relations in the organs of grapevines were assessed by recording changes in tissue water content, and in water- and osmotic-potential.

Tissue dehydration

Fresh roots, stems and leaves were weighed at the time of transfer (FW1) and during the treatment (FW2). The samples were dehydrated for 48 h at 80°C to obtain the dry weight (DW1 and DW2). The water loss percentage was calculated according to the following formula: 100× [(FW1 −DW1)/FW1] −[(FW2 −DW2)/FW2]. The dry weight of each organ was used to calculate metabolite contents [total soluble sugars (TSS), free proline, total free amino acids, thiobarbituric acid reactive substances (TBARS)].

Stem water potential

Water potential was measured on fresh stems (from which leaves and roots were removed) using a pressure chamber (model 603, PMS Instrument, Corvallis, Ore.) according to Schölander et al. (1965). The stem water potential corresponded to the appearance of the first sap droplet on the cut stem surface under increasing pressure. The data, obtained in bars, were converted into SI units (MPa).

Leaf osmotic potential

The cell sap was extracted from fully hydrated leaf blades after a freeze/thaw cycle in a 2 ml syringe. The osmotic potential of the sap was measured using an automatic micro-osmometer (Type 13 DR, Roebling, Berlin, Germany). The data, obtained in mosmol kg^{-1}, were converted into MPa; −2.5 MPa corresponding to 1,000 mosmol kg^{-1}. The osmotic potential measured in the culture medium did not change upon addition of FMX, regardless of the herbicide concentration.

Soluble sugars

Fresh plantlets were frozen in liquid N_2 and kept at −80°C for biochemical analysis. Leaves, stems and roots were ground separately at 4°C in a mortar in a 0.1 *M* phosphate buffer (pH 7.5). The homogenates were centrifuged for 15 min at 12,000 *g* and the supernatants were used for TSS determination: 200 μl supernatant was mixed with 1 ml anthrone-sulfuric reagent (0.1% anthrone and 0.1% thiourea in 12.5 *N* sulfuric acid) and incubated for 10 min at 100°C. After cooling, the absorbance was read at 625 nm (Yemm and Willis 1954) and results were expressed in mg glucose equivalents (g DW)$^{-1}$.

Free proline

A 200 μl aliquot of the previous potassium phosphate extract was mixed with 800 μl ninhydrin reagent [1% (w/v) ninhydrin in a 60% acetic acid solution (Magné and Larher 1992)]. The mixture was heated at 100°C for 20 min and then cooled in ice. Toluene (1 ml) was added and the sample was vigorously shaken for 15 s. The sample was placed in darkness at room temperature for at least 4 h. The absorbance of the upper phase was then read spectrophotometrically at 520 nm. Proline content was expressed in μmol (g DW)$^{-1}$.

Total free amino acids

A 200 μl aliquot of the potassium phosphate extract was mixed with 100 μl 0.2 *M* citrate buffer, pH 4.6 and 200 μl ninhydrin reagent [0.003% ascorbic acid and 0.96% (w/v) ninhydrin in ethylene glycol monomethyl ether (Magné and Larher 1992)]. The mixture was heated at 100°C for 20 min and then cooled in ice; 600 μl 60% ethanol were then added and the mixture was shaken. The absorbance was read at 570 nm using leucine as a standard.

Membrane alterations

Lipid peroxidation

Lipid peroxidation was evaluated by assaying the concentration of TBARS, determined according to Heath and Packer (1968). Fresh leaves were ground with Fontainebleau sand and trichloroacetic acid (TCA) (0.1% w/v). The homogenate was centrifuged at 4°C for 10 min at 12,000 *g*. One volume supernatant was mixed with 4 volumes 20% TCA containing 0.5% (w/v) 2-thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled in ice

and centrifuged at 10,000 *g* for 5 min. The absorbance of the supernatant was read spectrophotometrically at 532 nm and corrected by subtracting the value obtained at 600 nm (non-specific absorbance). Malondialdehyde (MDA) was used as a standard and the results were expressed in μmol MDA equivalents (g DW)$^{-1}$.

Relative electrolyte leakage

Fresh leaves were placed into 50 ml capped flasks containing 17 ml distilled water. After 2 h shaking (100 rpm) at room temperature, the conductivity of the solution ($C_{initial}$) was measured with a conductivity meter (model 150; Orion Research, Beverly, Mass.). The leaves were boiled in their immersion solution for 30 min, cooled at room temperature and total leaf electrolyte (C_{final}) was measured. The relative electrolyte leakage was calculated as the ratio of $C_{initial}$ over C_{final}.

Statistical analysis

Each measurement was repeated three times on at least six different plantlets and standard error was calculated.

Results

Water status

Water loss

Dehydration occurred in the aerial tissues of treated plantlets (Fig. 1). The leaf tissues lost water during the treatment as a function of the FMX concentration, with a maximal loss of 29% under 100 μM FMX at the end of the experiment (Fig. 1A). In the stem, water loss was also related to the FMX concentration, reaching a maximum of 12.4% after 2 weeks of treatment with 100 μM FMX (Fig. 1B). Nevertheless, during the third week of treatment, a partial rehydration was observed, especially at the low FMX concentrations. No significant water loss was found in the roots of the treated plantlets (Fig. 1C).

Stem water potential

The water potential of the control plantlets remained unchanged during the experiment, with values of about −0.3 MPa (Fig. 2). On the contrary, water potential decreased during FMX treatment as a function of herbicide concentration. Using 1 μM FMX, a 2-fold decrease occurred during the first 2 weeks of treatment. Thereafter, the stem water potential increased up to values close to the control. At higher concentrations, the water potential fell dramatically, reaching −1.76 and −2.54 MPa at the end of the treatments with 10 and 100 μM FMX, respectively. In these latter cases, the stem water potential did not recover.

Leaf osmotic potential

The osmotic potential of the control leaves remained unchanged after transfer, with a value of about −0.6 MPa (Fig. 3). On the contrary, it decreased during herbicide treatment as a function of the FMX concentration. reaching −1.33 MPa after 2 weeks of treatment with 1 μM FMX and increasing slightly thereafter. In the presence of 10 and 100 μM FMX, a 3- and 4-fold drop in osmotic potential, respectively, was found.

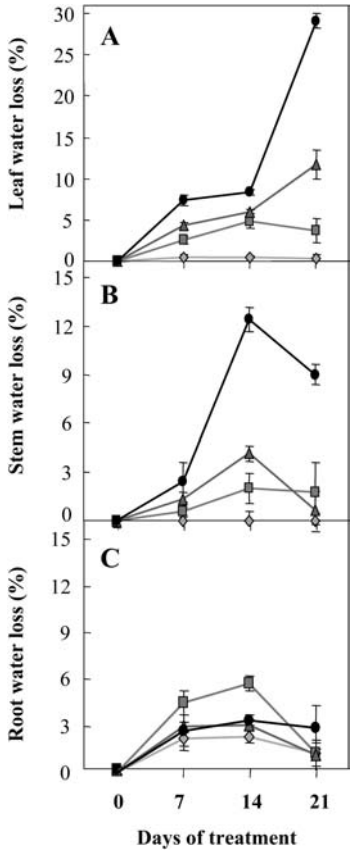

Fig. 1A–C Influence of flumioxazin (FMX) on grapevine water loss percentage. Six-week-old plantlets (day 0) were transferred for 3 weeks to new medium containing different concentrations of flumioxazin: ◆ 0 μM, ■ 1 μM, ▲ 10 μM, ● 100 μM. Water loss was investigated in the leaves (**A**), stem (**B**) and roots (**C**). Each value represents the mean of at least six measurements (±SE)

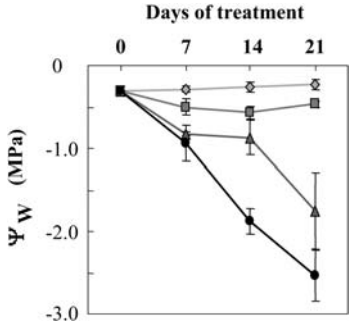

Fig. 2 Influence of FMX on grapevine stem water potential. Concentrations of FMX: ◆ 0 μM, ■ 1 μM, ▲ 10 μM, ● 100 μM. Each value represents the mean of at least six measurements (±SE)

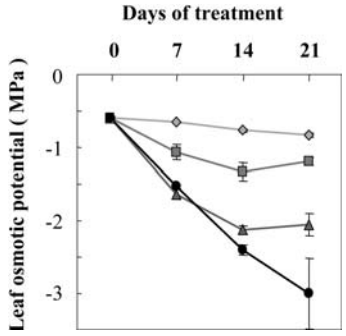

Fig. 3 Influence of FMX on grapevine leaf osmotic potential. Concentrations of FMX: ◆0 μM, ■1 μM, ▲10 μM, ●100 μM. Each value represents the mean of at least six measurements (±SE)

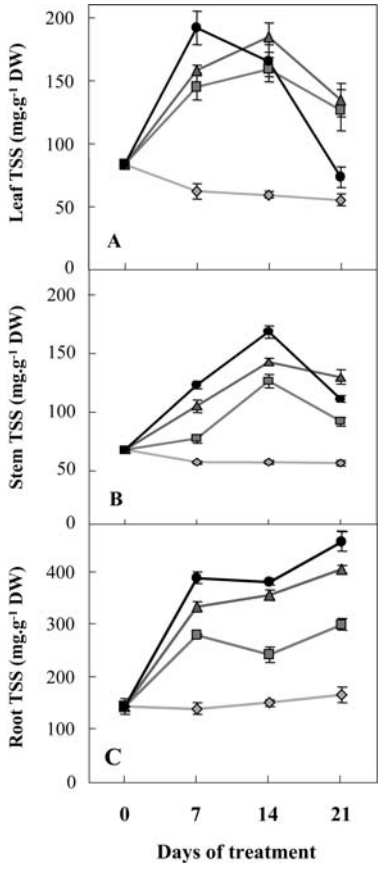

Fig. 4 Influence of FMX on grapevine leaf total soluble carbohydrate [total soluble sugars (TSS)] content was investigated in leaves (**A**), stem (**B**) and roots (**C**). Concentrations of FMX: ◆ 0 μM, ■ 1 μM, ▲ 10 μM, ● 100 μM. Each value represents the mean of at least six measurements (±SE)

Soluble sugars

In control plants, TSS content remained nearly unchanged during the experiment (Fig. 4). Upon FMX treatment, it increased greatly but transiently in the leaves, up to 209% for 100 μM FMX at day 7 (Fig. 4A). Later in the treatment, TSS content decreased but remained higher than that of control leaves. In the treated stems, the TSS content increased during the first 2 weeks (by 106% for 1 μM FMX to 183% for 100 μM FMX) and then declined during the third week, though the values remained higher than for the non-treated plants (Fig. 4B). Soluble carbohydrates accumulated in the roots of treated plants when the FMX concentration increased, particularly during the first week (Fig. 4C). At the end of the treatment, the root TSS content represented between 182% (1 μM FMX) and 278% (100 μM FMX) of the control.

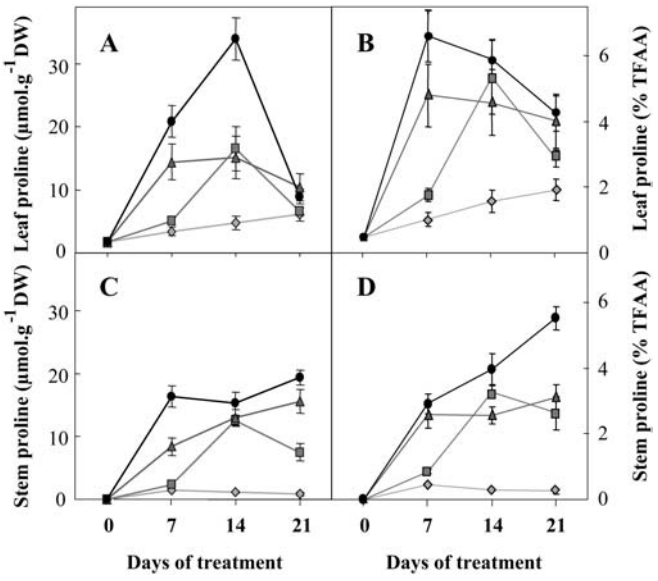

Fig. 5A–D Influence of FMX on free proline level in grapevine tissues. Concentrations of FMX: ◆ 0 μM, ■ 1 μM, ▲ 10 μM, ● 100 μM. **A** Leaf free proline. **B** Leaf free proline as a percentage of total free amino acids (TFAA). **C** Stem free proline. **D** Stem free proline as a percentage of TFAA. Each value represents the mean of at least six measurements (±SE)

Free proline

To evaluate specific fluctuations in proline content, both proline concentration and relative proportion of proline to total free amino acids (TFAA) are reported here. In the leaves, a transient increase in the proline level was found, followed by a decrease at the end of the treatment (Fig. 5A). The extent of the increase was related to the FMX concentration, ranging from 300% to 716% under 1 to 100 μM FMX, respectively. During the first 2 weeks, the contribution of proline to the TFAA pool increased, reaching 5–6.5% of the TFAA concentration, whereas it was only 1–1.5% in control plantlets (Fig. 5B). In the stem, the free proline level strongly increased under the three FMX concentrations tested (Fig. 5C). Proline accumulation under treatment occurred during the first week using 10 and 100 μM FMX, and during the second week using 1 μM FMX. At the end of the treatment, the proline content in the presence of 10 and 100 μM FMX was 18 and 22.2 times higher than that of the control,

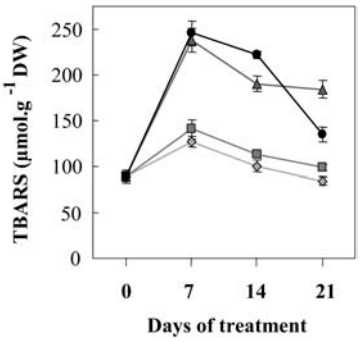

Fig. 6 Influence of FMX on grapevine leaf thiobarbituric acid reactive substances (TBARS). Concentrations of FMX: ◆ 0 *µM*, ■ 1 *µM*, ▲ 10 *µM*, ● 100 *µM*. Each value represents the mean of at least six measurements (±SE)

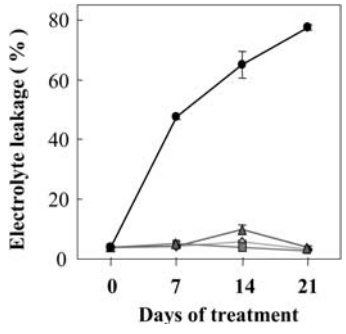

Fig. 7 Influence of FMX on grapevine leaf relative electrolyte leakage. Concentrations of FMX: ◆ 0 *µM*, ■ 1 *µM*, ▲ 10 *µM*, ● 100 *µM*. Each value represents the mean of at least six measurements (±SE)

respectively. Regarding the TFAA level, the percentage of free proline increased in treated grapevines, reaching 2–3% under 1 and 10 *µM* FMX and 5.5% under 100 *µM* FMX (Fig. 5D). Proline was not detected in grapevine roots (data not shown).

Membrane alteration

Lipid peroxidation

The level of TBARS was not markedly affected by the 1 *µM* FMX treatment and followed trends similar to those in the control plantlets (Fig. 6). Conversely, TBARS concentration in the leaf tissues strongly increased during the first week of treatment with 10 and 100 *µM* FMX, reaching about 200% of the control level. The TBARS concentration then decreased until the end of the treatment.

Relative electrolyte leakage

Electrolyte leakage from the leaf tissues remained approximately 4% after transfer on the control, 1 or 10 *µM* FMX medium (Fig. 7). In contrast, it increased dramatically during the 100 *µM* FMX treatment, i.e., after 1 week it reached 48%. After 3 weeks, the electrolyte leakage represented 77% of total electrolytes in 100 *µM*-treated plantlets and only 3% in control leaves or those treated with 1 or 10 *µM* FMX.

Discussion

The results presented here provide complementary information on the stress effects of the herbicide FMX on plant physiology in vitro (Saladin et al. 2003) that help to further understand (1) the mode of action of this herbicide on the non-target grapevine, and (2) the reaction of the plant to this compound at various concentrations.

In vineyards, FMX is applied to soil at a concentration of 4.5–5 m*M*, which is much higher than the concentration used in this study (1–100 *µM*). As FMX has been reported to be a molecule that is poorly mobile in soil, weeds are in contact with a higher herbicide concentration than we used. However, no information was available on the FMX concentration in contact with the grapevine root system in fields.

FMX treatment affected all the parameters related to plant stress that we evaluated. It induced a strong water loss and a parallel decrease in the water potential, indicating dehydration of the plantlet aerial organs. This dehydration process was slight in the presence of 1 *µM* FMX, but it appeared to be irreversible in grapevines grown under higher FMX concentrations. These results support previous work showing variable patterns of growth inhibition under FMX treatment, assessed by fresh biomass and stem height of grapevine plantlets (Saladin et al. 2003).

In parallel with aerial tissue dehydration, the leaves of FMX-treated plantlets exhibited a significant decline in osmotic potential in a dose-dependent manner. This result might be partially explained by the previously mentioned water loss, which led to a passive concentration of solutes in cells as reported by Balibrea et al. (1997). In addition, we found a strong increase in the soluble carbohydrate content in treated plants, although photosynthetic performance was markedly reduced (Saladin et al. 2003). The increased sucrose uptake from the medium is likely induced by this treatment. Sugar accumulation in higher plants under water stress has often been reported (Balibrea et al. 1997; Wang et al. 2000). In grapevine, it has been shown that, under drought, glucose and fructose may accumulate in immature leaves where they represent the main osmolytes (During 1984; Patakas and Noitsakis 2001). Accordingly, our results suggest that soluble carbohydrates may play a major role in active osmotic adjustment of grapevine leaves in response to FMX-induced water stress. On that point, one should note that the osmotic potential of the culture medium was not affected by the addition of FMX, even at the highest concentration. Therefore, the water stress described above

is most likely due to the herbicide and not to a decrease in the water availability in the medium.

In parallel with dehydration, a transient accumulation of TBARS was generated in the leaves of vines treated with 10 and 100 μM concentrations of FMX, indicating that lipid peroxidation occurred in cell membranes. Our results are in agreement with previous data obtained on PROTOX inhibitor herbicides, including oxyfluorfen or other diphenyl ethers (Wakabayashi and Böger 1995; Moreland 1999), in which the induced lipid peroxidation was proposed to be a consequence of a drop in carotenoid levels. Such a decline in carotenoid content of FMX-treated vines has been found recently in preliminary work by Saladin et al. (2003). Although FMX induced lipid peroxidation at a concentration as low as 10 μM, the modifications of the relative electrolyte leakage indicate that only the 100 μM FMX treatment caused a significant disturbance in membrane permeability. This discrepancy needs further investigation. It could be that lipid peroxidation is a more sensitive response to this herbicide than perturbation of membrane permeability. Electrolyte leakage could result from activation of transport proteins and not from non-specific leakage. On the other hand, the membrane structure of vine tissues treated with 10 μM FMX might be protected by the accumulated free proline and carbohydrates, as reported previously (Rudolph et al. 1986; Crowe et al. 1988).

In parallel to carbohydrates, proline has been reported to accumulate in plant tissues in response to a number of stress conditions including drought, salinity, low temperatures, air pollution or heavy metals (Greenway and Munns 1980; Aspinall and Paleg 1981; Rhodes 1987). In our experiment, free proline specifically accumulated in the aerial tissues during the first 2 weeks of FMX treatment. This accumulation could not be due to a translocation from the roots because proline was not detected in the roots. In addition, the increase in the free proline contribution in the TFAA pool was not due to a global decrease in TFAA concentration. Thus, the increase of proline observed in our study might result from de novo synthesis and/or inhibition of catabolism (Verma 1999), together with the water stress generated by FMX treatment. The proline accumulation, generally in the cytosol, might be involved in the cellular osmotic adjustment (Yoshiba et al. 1997; Watanabe et al. 2000).

Considering the data reported both here and in a previous paper (Saladin et al. 2003), we can provide an overview of FMX action on in vitro-grown grapevine plantlets that was not given by the manufacturer: (1) despite having been demonstrated to be a pre-emergence herbicide by contact with growing seedlings (Tomlin 2000), FMX also penetrates the plant via the roots; (2) FMX generates a specific chemical stress as revealed by dramatic changes in water status, lipid peroxidation, as well as the accumulation of proline and carbohydrates; (3) despite being a PROTOX inhibitor, FMX induces cell damage, thus altering membranes, and (4) according to its target FMX perturbs carbohydrate metabolism and photosynthesis, leading to a significant reduction in growth. Owing to the toxic effects of FMX on the non-target in vitro-grown plantlets, additional analysis is necessary to investigate plants grown in vineyards following FMX treatments. Nevertheless, plantlets seem to be capable of tolerating moderate concentrations of FMX (1 μM) by accumulating carbohydrates that are known to protect cell membranes against intense dehydration (Hoekstra et al. 2001).

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