Abstract The impact of flumioxazin herbicide on in vitro-grown grapevine (Vitis vinifera L. cv. Chardonnay) was investigated. The herbicide treatments (1, 10 or 100 μM flumioxazin in MM medium) had a negative impact on photosynthesis, as revealed by a reduction in foliar chlorophyll and carotenoid contents, gas exchanges and alteration in plastid structure and, consequently, resulted in a strong inhibition of plantlet growth. Surprisingly, soluble sugars and starch accumulated in all organs, suggesting a stimulation of sugar uptake from the medium. Moreover, photosynthetic activity and starch content partially recovered within 3 weeks of treatment at the weakest herbicide concentration. These results provide new insights into the physiological responses of non-target crops to flumioxazin, showing that flumioxazin is active in photosynthetic tissues of the non-target grapevine via root uptake, which is contrary to what is mentioned in the literature, and that the in vitro-grown plantlet is a good model for investigating the physiological effects of pesticides on crop species.

Keywords Carbohydrates · Flumioxazin · Grapevine · Herbicide · Photosynthesis

Introduction

Herbicides are widely used to protect crops against adventitious plants. Nevertheless, a massive introduction of these molecules into the fields can generate negative effects on the environment. Since increasingly more consumers are becoming aware of agricultural practices and their impact on the environment and food quality, pesticide toxicity for non-target crop species is a topic that needs to be investigated.

It has been shown that growth of several crop species, including grapevine, may be affected by herbicides such as 2,4-dichlorophenolxyacetic acid (2,4-D), glyphosate, chlorsulfuron, diuron or trichlroracetate (Bhatti et al. 1997, 1998; Itoh and Manabe 1997; Radetski et al. 2000), even though they are not targeted by the treatments. These herbicides induce leaf necrosis, an increase in stomatal resistance, inhibition of shoot growth, a decrease in germination, an increase in levels of active oxygen species and a reduction of net photosynthesis. However, while little is known about the effects of newly synthesized herbicides on crop species, the presence of such molecules in the foliage of non-target crops and in the soil has been reported (Jame et al. 1999).

Flumioxazin (fmx), or 2-[7-fluoro-3,4-dihydro-3-oxo-4-(2-propynyl)-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione (Bhowmik 2000), is a soil-applied herbicide of the N-phenylphthalimide family, which has recently been allowed for use in French vineyards. This pre-emergence herbicide is absorbed by germinating seedlings and stops the first stages of development (Labonne and Capou 1998). Weed species then start to bleach and rapidly die off. Fmx is an effective inhibitor of protoporphyrinogen IX oxidase (protox), an enzyme involved in both chlorophyll and heme biosynthesis through the transformation of protoporphyrinogen IX to protoporphyrin IX. Protoporphyrinogen IX oxidase (protox) inhibitors such as fmx are thought to induce a diffusion of protoporphyrinogen IX away from the plastids (Hess 1993). Another protox-like enzymatic activity associated with the plasma membrane would then oxidize protoporphyrinogen IX to protoporphyrin IX (Lee and Duke 1994), outside the plastids, interacting with light and oxygen to produce singlet oxygen. This activated oxygen species is known to be an initiating factor of lipid peroxidation, which leads to senescence.

Despite the wide use of protox inhibitors in crop culture, their mode of action remains poorly understood (Theodoridis et al. 2000). In addition, most of the
information related to their activity has been obtained from studies on diphenyl ether herbicides (Hess 1993; Wakabayashi and Boger 1995; Moreland 1999), whereas little is known about the phthalimides (Labonne and Capou 1998; Tomlin 2000).

Costa and Spitz (1997) and Sanità di Toppi et al. (1998) used plants grown in vitro to study the effects of heavy metals on plant physiology. In vitro-grown plants represent an interesting experimental model to measure the effects of various pollutants. Plantlets grown under entirely controlled conditions provide the means to evaluate accurately the influence of a single parameter on plant physiology. For example, it has recently been shown that an excess of copper affects the nitrogen metabolism of grapevine (Llorens et al. 2000). Therefore, we have designed our study of the effects of fmx on grapevine physiology using in vitro-grown plants. In order to assess the influence of this molecule widely used in vineyards, we investigated several aspects of plant physiology. The aim of the work presented here was to evaluate more precisely the effects of fmx on carbohydrate physiology by measuring fluctuations in photosynthesis, alterations in the photosynthetic apparatus and the parallel variations in pigments and carbohydrates.

**Materials and methods**

**Plant material**

Microcuttings of *Vitis vinifera* L. cv. Chardonnay (clone 7535) were grown in culture tubes on 10 ml of MM medium (Martin et al. 1987) at 26°C and an 80–85% relative humidity, under a 16/8-h (day/night) photoperiod, with light supplied at a photon flux density 1987) at 26°C and an 80–85% relative humidity, under a 16/8-h photoperiod, with light supplied at a photon flux density of 1,000 µmol m⁻² s⁻¹. After 6 weeks, the plantlets were transferred onto new MM media containing 0 (control), 1, 10 or 100 µM of the fmx herbicide. Samples were collected before the transfer (day 0) and after 7, 14 and 21 days of treatment.

**Gas exchange measurements**

Fresh plantlets collected from the culture tubes were immediately used for gas exchange measurements. The maximum rate of CO₂ assimilation was measured using a Li-Cor 6200 portable photosynthetic system (Li-Cor, Lincoln, Neb.) with a 250-ml gas exchange chamber. Measurements were performed with a saturating irradiance, a relative humidity of 65–75%, an air CO₂ concentration of 400 µl l⁻¹ and a flow rate of 1,000 µmol min⁻¹. O₂ production was measured with an oxygen electrode (Hansatech, Kings Lynn, UK). Three detached leaves from a single plantlet were placed in the electrode chamber. CO₂ was maintained in excess using 2 M potassium carbonate/potassium bicarbonate buffer, pH 9.3. The chamber was illuminated with a white light source (900 µmol photons m⁻² s⁻¹) during the measurements. The electrode buffer contained saturated potassium chloride. Leaf photosynthetic pigments

The whole extraction procedure was carried out under low light intensity in order to minimize chlorophyll alterations. All of the leaves of a plantlet were ground at 4°C with Fontainebleau sand in 80% (v/v) acetone, in the presence of 50 mg MgCO₃ to prevent chlorophyll acidification. The crude extract was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was kept at 4°C. The pellet was then homogenized twice with 80% (v/v) acetone and centrifuged at 10,000 g for 10 min at 4°C. The three supernatants were pooled, and the chlorophyll and carotenoid concentrations were estimated spectrophotometrically according to the absorbance coefficients determined by Lichtenthaler (1987).

**Transmission electron microscopy**

Leaves were fixed for 24 h at room temperature in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.24) with 2% (w/v) sucrose and 0.1% (v/v) Tween 20. After three rinses (5 min each) with the phosphate buffer and 2% (w/v) sucrose, leaves were post-fixed for 4 h in 1% (w/v) osmium tetroxide in the phosphate buffer with 2% (w/v) sucrose. The leaves were then dehydrated in an alcohol series, transferred to acetone and embedded in araldite. Ultra-thin sections were collected on 150-mesh copper grids with a diamond knife on a Reichert Omu2 ultratome, stained with uranyl acetate/lead citrate and examined on a JEOL JSM 201 electron microscope at 80 kV.

**Carbohydrate content**

**Extraction**

Leaves, stems and roots were ground separately at 4°C in a mortar containing 0.1 M phosphate buffer (pH 7.5). The homogenates were centrifuged at 12,000 g for 15 min, and the supernatants were used for soluble sugar determination, whereas the pellets were kept for starch analysis.

**Starch**

The pellet collected following the extraction of total soluble sugars was resuspended in dimethylsulfoxide/8 N hydrochloric acid mixture (4:1, v/v). Starch was dissolved for 30 min at 60°C under agitation. After centrifugation for 15 min at 12,000 g, 100 µl of the supernatant was mixed with 100 µl of trichloroacetic acid solution (0.006% KI and 0.003% I₂ in 0.05 N HCl) and 1 ml of distilled water. The absorbance was read at 600 nm after 15 min at room temperature.

**Soluble carbohydrates**

Samples of 300 µl were used to determine soluble sugar concentration according to Bergmeyer (1974) using a Boehringer Mannheim enzymatic kit (R-Biopharm, Darmstadt, Germany). The d-glucose was phosphorylated and oxidized in the presence of nicotinamide dinucleotide phosphate (NADPH) to form both glucose-6-phosphate and NADH. The amount of NADPH was finally determined by means of its absorbance at 340 nm. For fructose determination, fructose in the homogenate was phosphorylated and converted to glucose-6-phosphate. Glucose-6-phosphate was tested as described above. For sucrose determination, sucrose in the homogenate was hydrolyzed to d-glucose and d-fructose in the presence of a β-fructosidase. The concentration of d-glucose was then determined as described above and a blank was performed without β-fructosidase.

For each experimental condition, the assay was performed three times from three different samples. For each homogenate, three independent readings were carried out. Chemicals for the sugar...
determination were obtained from Boehringer (Mannheim, Germany). Results are given in micromoles per gram dry weight.

Statistical analysis
Each measurement was repeated three times on at least six different plantlets, and standard errors were calculated.

Results

Growth

The fmx treatment induced a dramatic decrease in stem growth in a dose-dependent manner (Fig. 1A). The inhibition of shoot growth in treated plantlets was accompanied by browning of the roots and, to a lesser extent, reddening of the stem. During the first week, stems of the non-treated plants grew by 47% compared to the 23%, 14% and 2% for plants cultivated in the presence of 1, 10 and 100 \( \mu M \) fmx, respectively (Fig. 1B). Stem growth slowed down during the 21 days of the experiment. Stem height increased by 121% in the control during the experiment, while it increased by 59%, 23% and 14% for plants treated with 1, 10 and 100 \( \mu M \) fmx, respectively.

The inhibitory effect of fmx on plantlet growth was also revealed by a decrease in both shoot and root biomass (Fig. 2). After 3 weeks of treatment, the shoot fresh weight for plants treated with 1 \( \mu M \) fmx corresponded to 75% of that of the control (Fig. 2A). At higher concentrations (10 \( \mu M \) and 100 \( \mu M \)), the shoot weight reached 52% and 38%, respectively, of that of the control plants. The effect of fmx on dry weight was less marked (Fig. 2B). Shoot dry weight was quite similar in the control and in the treated plants. During the experiment, root fresh weight increased regularly in the control plants (Fig. 2C). In the presence of fmx, root fresh weight decreased during the first 2 weeks irrespective of the herbicide concentration, reaching about 60% of that of the control, then it recovered. With regards to the dry weight, the effect of herbicide concentration was more obvious (Fig. 2D): at day 21, the root dry weight of treated plants was 20–33% lower than that of the control depending on the concentration applied.

Gas exchanges

In the control plant, the maximum rate of \( \text{CO}_2 \) assimilation remained unchanged during the experiment except for a slight increase after 2 weeks (Fig. 3A). Whatever the fmx concentration, the most drastic effect occurred after 1 week of treatment, when a 30–70% decrease in \( \text{CO}_2 \) fixation – depending on the fmx concentration – was induced. At the end of the 1 \( \mu M \) treatment, the rate of \( \text{CO}_2 \)
assimilation was not significantly different from that of the control, whereas it decreased down to 32% and 8% at 10 \mu M and 100 \mu M fmx, respectively.

In the control plants, O$_2$ production decreased during the first 2 weeks after transfer (Fig. 3B). This result was reinforced in the presence of fmx, especially with the 10 \mu M and 100 \mu M treatments, which respectively generated 64% and 81% reductions. Using 1 \mu M fmx, the O$_2$ production was slightly reduced, but at the end of this treatment, vines produced O$_2$ at a level similar to that of the control.

Leaf photosynthetic pigments

Fmx induced a reduction in leaf chlorophyll content (Fig. 4A). During the first 2 weeks, the chlorophyll content of the non-treated plants increased by 18%. In the presence of the herbicide, there was a rapid decline in chlorophyll content during the first week of treatment that was correlated to the concentration of fmx: the chlorophyll contents were reduced by 26%, 35% and 50% using 1, 10 and 100 \mu M fmx, respectively. Leaf chlorophyll content was not significantly modified during the second week of treatment, but it decreased again during the last week. The strongest effect was observed with 100 \mu M fmx, resulting in a 80% chlorophyll loss.

Leaf carotenoid content decreased during the fmx treatment (Fig. 4B). During the first week of treatment, a similar effect was observed with the three tested fmx concentrations. During the last 2 weeks of the treatment with 1 \mu M fmx, the carotenoid content remained unchanged. However, in the presence of 10 \mu M and 100 \mu M fmx, the carotenoid content further declined, reaching respectively 57% and 20% of the control level after 3 weeks of treatment.

Chloroplast ultrastructure

In the leaves of non-treated plants, the chloroplasts had an elongated shape and were 7.01\pm2.42 \mu m in length and 2.38\pm0.7 \mu m wide (Fig. 5A). The plastids included well-developed thylakoids organized in grana containing up to 25 sacculles (Fig. 5B). Small starch grains and plastoglobules were present in most of chloroplasts. Under the 100 \mu M fmx treatment, the most important effect of the herbicide was a spectacular development of starch grains (Fig. 5C). The plastids were more spherical in shape and were 4.56\pm0.47 \mu m long and 3.14\pm0.66 \mu m wide. The membranes were not structurally altered, but the grana were less developed and thylakoid disorganization had occurred (Fig. 5D): spaces were detected between thylakoids, and the positioning of the thylakoids was no longer parallel. Moreover, the plastoglobules were significantly larger in plastids of the treated plants than in those of the control.

Carbohydrates

No starch was found in the roots and shoots of control and treated plants. In the leaves, the starch content decreased slightly during the experiment in both control and 1 \mu M fmx-treated plants (Fig. 6). On the contrary, high concentrations of fmx induced an accumulation of starch that was detectable after 7 days: starch content increased by 111% and 217% in the presence of 10 \mu M fmx and 100 \mu M fmx, respectively. During the last week, starch content decreased by 24% using 10 \mu M fmx, whereas it increased by 47% in the 100 \mu M fmx-treated plants.

Leaf sucrose content increased considerably during the first week of treatment at all three fmx concentrations (Fig. 7A). The increase ranged from 395% to 520% at 1 \mu M and 100 \mu M, respectively. The sucrose content subsequently declined until the end of the treatment but remained higher than in the control leaves. For all three fmx concentrations, leaf glucose (Fig. 7B) and fructose (Fig. 7C) contents strongly increased during the second week of treatment, whereas they decreased during the last week of treatment, particularly in the presence of 100 \mu M fmx.

In the stem, sucrose content increased during the first week of treatment, reaching 154% and 230% of the control level at 1 \mu M and 100 \mu M, respectively (Fig. 7D). It then fell between day 7 and day 14, subsequently
increasing again during the third week of treatment for all three fmx treatments. For all the fmx concentrations, glucose and fructose concentrations fluctuated in parallel, accumulating in the stem during the second week. The hexose contents then dropped to values close to those of the control plants (Fig. 7E, F).

Relative to the control, root sucrose content increased during the first and the third weeks of fmx treatment but decreased during the second week (Fig. 7G). Glucose and fructose contents increased throughout treatment as a function of fmx concentration (Fig. 7H, I). The increase was the strongest during the second week of treatment. At the end of the treatment, the glucose content for the 10 μM and 100 μM fmx treatments represented 300% and 400% of the control level, respectively, whereas the fructose content corresponded to 565% and 835% of the control level.

Fig. 5A–D In vitro influence of fmx on leaf plastid ultrastructure. A Untreated plants. Plastids had an elongated shape and enclosed a low amount of starch (arrowheads). N Nucleus, V vacuole. Magnification: 5,000×. B Untreated plants. A higher magnification showing a plastid with well-organized thylakoids and grana (arrowheads), no starch and a few plastoglobules (white stars). P Plastid. Magnification: 25,000×. C Plants treated with 100 μM fmx for 21 days. Plastids were spherical and accumulated huge starch grains (S). Magnification: 5,000×. D Plants treated with 100 μM fmx for 21 days. Higher magnification showing thylakoid disorganization (arrowheads), accumulation of plastoglobules (white stars) and starch grains. Magnification: 22,000×

Fig. 6 In vitro influence of different concentrations of fmx on grapevine leaf starch content. Solid diamond 0 μM, solid square 1 μM, solid triangle 10 μM, solid circle 100 μM
Discussion

These results provide new insights into the effects of fmx herbicide on grapevine physiology through the analysis of many parameters. This herbicide causes an alteration in the photosynthetic apparatus, a reduction in net photosynthesis and a serious inhibition of grapevine growth. In that respect, fmx altered both fresh and dry matter accumulations in vine roots. However, it impaired only fresh biomass production in shoot and leaf tissues, indicating that the herbicide caused a decrease in the water content of these aerial tissues concurrently with a strong accumulation of carbohydrates.

Flumioxazin was found to affect a number of parameters related to photosynthesis. The in vitro application of fmx to grapevine plantlets induced an alteration to the plastid structure. This disorganization of internal photosynthetic membranes is in accordance with the membrane alteration that was observed upon treatment with various photobleaching herbicides (La Rocca et al. 2000a, 2000b), although the modifications we observed may have originated from a large accumulation of carbohydrates. Consequently, fmx inhibited both CO₂ assimilation and O₂ production.

However, in the presence of 1 µM fmx, a slight decrease in photosynthesis during the first week of treatment was followed by the recovery of gas exchanges at the end of the experiment. This indicates that in vitro-grown plantlets may tolerate a low herbicide concentration after 1 week of adaptation.

In accordance with the information provided by the manufacturer, fmx induced a marked decline in chlorophyll content. This trait was revealed by leaf bleaching and necrosis areas spreading gradually as a function of fmx concentration and time of exposure. In addition, the amount of carotenoids in the leaves was reduced to a similar extent to that of the chlorophylls, although the synthesis of carotenoids is not the target of fmx. In fact, fmx has been shown to cause an overproduction of excited chlorophyll molecules and, subsequently, singlet oxygen, thus leading to a strong oxidative stress (Hess 1993). Such an oxidative process might be responsible for the decrease in carotenoid content, through carotenoid oxidation, as it has been reported recently in other species (Munné-Bosch and Alegre 2000).

Fmx induced an accumulation of soluble sugars in every organ of the plantlets. This result is in agreement with those observed under many stress conditions, including water deficit (Pelah et al. 1997), suboptimal temperatures (Adachi et al. 2000; Savitch et al. 2000), cadmium treatment (Costa and Spitz 1997), fungal infections (Goicoechea et al. 2000) and salt or osmotic stress (Balibrea et al. 1997; Kerepesi and Galiba 2000). Since photosynthesis was reduced, the transient accumulation of sucrose in the roots, and to a lower extent in the stems, indicates that sucrose uptake from the medium was highly stimulated upon fmx treatment. The subsequent increase in glucose and fructose contents in all of the vine tissues might be due to hydrolysis of previously accumulated sucrose. Fmx induced starch accumulation in the vine leaves. The accumulation of starch under abiotic stress has been reported in this species (Aït Barka and Audran 1996). In our experiment, such an accumulation occurred under conditions inducing impaired photoassimilation and might be due to the storage of the up-taken sugars.

The data presented here provides new insights into the mechanism of fmx action on plants. Our results were
obtained using fmx concentrations (1–100 μM) much lower than that commonly used in vineyards (approx. 4.5 mM) because in vitro-grown plantlets rapidly died (3–4 days) using a fmx concentration higher than 200 μM. However, in natural conditions, it is generally assumed that fmx is poorly mobile in the soil (Labonne and Capou 1998), so it may not reach crop tissues unless climatic accidents (violent rainfalls, storms) occur. In that respect, fmx is assumed to act on plant tissues by contact only, not systemically. Conversely, our results indicate that the fmx herbicide may be taken up by grapevine roots, then translocated to the upper tissues where it acts on growth and photosynthesis.

The objective of our investigation was to examine possible responses of non-target grapevine to a herbicide recently used in vineyards on an experimental model. It appears that the in vitro culture provides an efficient tool by which to investigate physiological responses of grapevine to fmx herbicide. In particular, in the presence of 1 μM fmx, a transient alteration in some physiological parameters (gas exchanges, carbohydrate contents) was followed by the recovery of these same parameters at the end of the experiment, indicating that in vitro-grown vine seems capable of adapting to a low fmx concentration. This model might, therefore, be of great interest for investigating grapevine physiology under various conditions.

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References