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Effects of Flumioxazin Herbicide on Carbon Nutrition of *Vitis vinifera* L.

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To evaluate the impact of the herbicide flumioxazin (fmx) on nontarget grapevines, its effects were assessed on fruiting cuttings and field-grown plants. The stress caused by the herbicide differed according to the grapevine model. In cuttings, leaf gas exchange and photosynthetic pigment levels as well as hexose contents decreased, whereas sucrose and starch accumulated, suggesting an inhibition of photosynthesis and an increase of carbohydrate reserves as a response to the fmx-induced stress. Paradoxically, in the field-grown grapevine leaves, fmx caused a stimulation of photosynthesis, an accumulation of photosynthetic pigments and monosaccharides, in parallel with a mobilization of sucrose and starch. These results suggest that fmx reaches grapevine leaves via root uptake and has prolonged effects. In cuttings, fmx generated a toxic effect related to its target, whereas in field-grown plants, fmx had rather positive physiological effects and acts as a signal further stimulating photosynthesis and related parameters.

KEYWORDS: Carbohydrates; flumioxazin; fruiting cutting; grapevine; herbicide; photosynthesis; vineyard

INTRODUCTION

Herbicides are widely used in agriculture to control weeds even though some of them may have negative effects on crop growth. Herbicides accumulate not only in the environment but also in crops (*1*). Moreover, they may alter crop physiology by inhibiting plant growth, delaying development, decreasing seed germination, and reducing leaf area in *Brassica campestris* L. or *Glycine max* L. (*2*, *3*). Some herbicides reduce photosynthesis in *Phaseolus vulgaris* L. (*4*), and cause membrane alterations in *Glycine max* L. and *Cucumis sativus* L., leading to cell death (*5*, *6*). Consequently, herbicide treatments can cause a yield reduction, reaching 20% in *Cucurbita* spp. and up to 50% in *Medicago sativa* L. (*1*, *7*, *8*). In grapevines, it has been reported that herbicide exposure can generate a reduction of both leaf area and internode length, an inhibition of photosynthesis, as well as an increase in stomatal resistance and chlorotic area in leaves (*9*−*13*).

Flumioxazin, or 2-[7-fluoro-3,4-dihydro-3-oxo-4-(2-propynyl)-2*H*-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione (*14*), is a *N*-phenylphthalimide herbicide used in French vineyards since 1998. This preemergence herbicide is applied on soil at the end of winter at a concentration of 5 mM. It inhibits protoporphyrinogen IX oxidase (protox), an enzyme involved in the biosynthetic pathway of both chlorophyll and heme. Protoporphyrinogen IX accumulates in plastids and then diffuses through the plastidial membranes into the cytosol, where it is oxidized to protoporphyrin IX by a plasma membrane-bound protox (*15*). The protoporphyrin IX reacts with light to produce singlet oxygen, leading to lipid peroxidation and the destruction of cellular components (*6*). Despite the fact that the mode of action of the protoporphyrinogen IX oxidase inhibitors and their effects on crops have been described in detail, most of the work with fmx reported its efficacy on weed control or its negative impact on crop yield (*16*, *17*), which represents up to a 30% yield reduction in *Arachis hypogaea* L. (*18*, *19*). Little information is available on the effect of fmx on crop physiology (*20*), especially in grapevine, although it is one of the most frequently used herbicides in French vineyards. We recently showed that fmx dramatically affects grapevine physiology in vitro (*21*, *22*). Various concentrations of this herbicide have a negative impact on vine plantlet leaf growth, as revealed by tissue dehydration and cell membrane alteration, a decrease in osmotic potential, and an accumulation of proline (*21*). Moreover, fmx treatment results in a reduction of plantlet growth and photosynthesis and induces perturbation in leaf carbohydrate partitioning (*22*). However, these results were obtained using juvenile plantlets grown in vitro and thus have to be considered cautiously before being extended to the whole plant.

Therefore, to determine if fmx has similar consequences on entire plants, two grapevine models were tested: fruiting cuttings and vineyard-grown plants. The aim of this work was (1) to characterize the effects of fmx on grapevine physiology and (2) to determine whether fruiting cuttings can be considered as a good model for the behavior of plants grown in the vineyard. The physiological impact of fmx was investigated using

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parameters related to photosynthesis, which has a direct impact on yield (*22*).

MATERIALS AND METHODS

Plant Materials. *Vineyard Grown Plants.* The field study was performed in a French vineyard (Reuil sur Marne, Champagne) on 3 cvs. grown in this area (Chardonnay CH clone 7535, Pinot Meunier PM, and Pinot noir PN). In February 2002, the commercial herbicide Pledge was sprayed at a concentration of 5 mM fmx on the soil at the base of dormant vines. The assessment of fmx effect was performed 5 months later, when the vines were at the fruit set stage for both control and treated plants. The third leaf (from bottom) of 10 different plants was used for photosynthesis measurements, as it is considered as representative of the carbohydrate physiology in mature grapevine leaves. The leaves were then collected for biochemical analysis. Leaves from untreated plants were simultaneously harvested and used as negative controls.

Fruiting Cuttings. Canes of *Vitis vinifera* L. cv. Chardonnay (clone 7535) were collected in winter and kept in a cold room at 4 °C for a minimum of 2 weeks. They were then cut to obtain two consecutive fertile buds and one sterile bud (*23*, *24*). The sterile bud was soaked for 3 min in a 0.1% (w/v) aqueous 3-indolylbutyric acid solution in order to stimulate rhizogenesis. Then, the cuttings were placed in 300 mL pots containing 25% blond turf, 50% brown turf, and 25% clay (105c soil, Agrofino, Arles, France) at 25 °C and 80% relative humidity in the greenhouse, with a 16-h photoperiod at a photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1}. Plants were daily irrigated with a nutrient solution optimized for grapevine culture (*25*), and four leaves were kept on each cutting. After 8 weeks, roots were developed, and the commercial herbicide was sprayed on the soil at different concentrations of the active molecule fmx: 0.1, 5 (concentration recommended by the manufacturer), or 10 mM. The control treatment consisted in replacing the herbicide application by a water spray. The third developed leaf of the cuttings was collected at the time of the treatment (day 0) and after 7, 14, or 21 days.

Photosynthesis Measurements. The maximum rate of net CO_2 assimilation was measured using a Li-6400 portable photosynthetic system (Li-Cor Inc., Lincoln, NE). Measurements were performed between 10 and 12 a.m. (*26*) under optimal growth conditions: 25 °C, 70% RH, 6 cm^2 of sun-exposed leaf surface. The CO_2 fixation was carried out at optimal light intensity (1000 and 1800 μmol photons m^{-2} s^{-1} in greenhouse and in vineyards, respectively). Results were expressed in μmol m^{-2} s^{-1}.

Leaf Photosynthetic Pigments. The entire extraction procedure was carried out under low light intensity at 4 °C in order to minimize chlorophyll alteration. Leaves were ground with Fontainebleau sand in 80% (v/v) acetone, in the presence of 0.5% (w/v) $MgCO_3$ 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 re-extracted 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 (*27*). Results were expressed in mg g^{-1} DW.

Carbohydrates. *Extraction Procedure*. Leaves were ground at 4 °C in a mortar containing 0.1 M phosphate buffer (pH 7.5). The homogenate was centrifuged at 12 000*g* for 15 min at 4 °C and the supernatants were used for soluble sugar determination whereas the pellet was kept for starch analysis.

Soluble Carbohydrates. Samples of 100 μL were used to determine individual soluble carbohydrates using a Boehringer Mannheim enzymatic kit (R−Biopharm GmbH, Darmstadt, Germany). D-Glucose was phosphorylated and oxidized in the presence of nicotinamide dinucleotide phosphate (NADP) to form both gluconate-6-phosphate and NADPH. The amount of NADPH was determined by means of its absorbance at 340 nm. Fructose was phosphorylated and converted to glucose-6-phosphate, which was assayed as described above. Sucrose 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. Results were expressed in mg g^{-1} DW.

Starch. The pellet collected after the extraction of soluble carbohydrates was resuspended in dimethyl sulfoxide−8 N hydrochloric acid mixture (4:1 v/v). Starch was dissolved by agitation for 30 min at 60 °C. After centrifugation at 20 °C for 15 min at 12 000*g*, 100 μL of the supernatant was mixed with 100 μL of iodine−HCl solution (0.06% KI and 0.003% I_2 in 0.05 N HCl) and 1 mL of distilled water. The absorbance was read at 600 nm after 15 min at room temperature, and results were expressed in mg g^{-1} DW.

Statistical Analysis. Each data point was the mean for the third leaf of 10 different plants (fruiting cuttings or field-grown grapevines). The results presented are mean values ± standard errors (SE). Standard analysis of variance (*t* test) was used to assess the significance of the treatment means at $P < 0.05$, 0.01, or 0.001 level.

Table 1. Influence of fmx on CO_2 Fixation in Fruiting Cuttings

fmx treatment (mM)	CO_2 fixation:[a] at days of treatment			
	0	7	14	21
0 (control)	12.5 ± 0.49	15.12 ± 1.29	15.10 ± 0.92	15.36 ± 0.97
0.1		13.79 ± 0.04*	14.25 ± 0.60^{ns}	14.50 ± 0.70^{ns}
5		12.13 ± 0.56***	12.15 ± 0.23***	12.53 ± 0.90**
10		13.90 ± 0.50*	12.91 ± 0.43**	12.56 ± 0.01**

[a] Results were expressed in μmol m^{-2} s^{-1}. Data are means ± Se ($n = 10$). Levels of significance were represented by * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$ and ^{ns} (not significant).

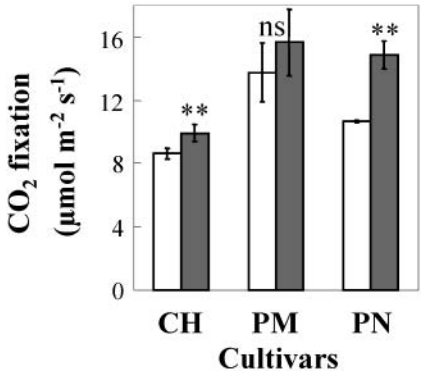

Figure 1. Influence of fmx on CO_2 fixation in the vineyard. The control and treated plants were represented in white and gray, respectively. CH, Chardonnay; PM, Pinot Meunier; PN, Pinot noir. Data are means ± SE ($n = 10$). Levels of significance were represented by * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$ and ^{ns} (not significant).

RESULTS

Gas Exchanges. In the fruiting cuttings, fmx caused a significant decrease in CO_2 assimilation after 7 days of treatment. At the end of the experiment, the decrease represented up to 20% at 5 and 10 mM fmx, whereas a recovery was observed at 0.1 mM fmx after 14 days (**Table 1**). In contrast, plants grown in the vineyard and treated with fmx exhibited a higher CO_2 fixation (**Figure 1**). Among the 3 tested cvs., PN appeared to be the most sensitive cv. to fmx, as CO_2 fixation increased by 39%, whereas it increased by 13−15% in the 2 others cvs., though the difference was not significant in PM.

Photosynthetic Pigments. Fmx had opposite effects on cuttings and plants grown in vineyards. In cuttings, after 7 days of treatment, the chlorophyll level of cutting leaves was slightly reduced at 0.1 mM fmx, but it rapidly recovered (**Table 2**). At high concentrations, fmx caused a decrease in leaf chlorophyll content, especially after 21 days of treatment. Indeed, the chlorophyll concentration was, respectively, 28 and 75% lower

Table 2. Influence of fmx on Chlorophyll and Carotenoid Concentrations in Fruiting Cuttings

fmx treatment (mM)	concentration:[a] at days of treatment			
	0	7	14	21
		Chlorophyll Concentration		
0 (control)	7.19 ± 0.08	10.15 ± 1.15	9.19 ± 0.49	10.28 ± 0.66
0.1		8.37 ± 1.37^{ns}	10.37 ± 0.65^{ns}	11.25 ± 2.47^{ns}
5		7.00 ± 0.29***	8.57 ± 0.87^{ns}	7.38 ± 0.35**
10		8.73 ± 0.94^{ns}	8.29 ± 0.96^{ns}	2.60 ± 0.05***
		Carotenoid Concentration		
0 (control)	1.88 ± 0.10	1.90 ± 0.36	2.46 ± 0.17	2.36 ± 0.56
0.1		2.38 ± 0.03*	1.85 ± 0.21^{ns}	2.11 ± 0.14^{ns}
5		1.83 ± 0.09^{ns}	2.11 ± 0.15^{ns}	1.53 ± 0.07***
10		2.34 ± 0.46^{ns}	1.74 ± 0.21*	0.78 ± 0.14***

[a] Results were expressed in mg g^{-1} DW. Data are means ± SE (n = 10). Levels of significance were represented by * at P < 0.05, ** at P < 0.01, *** at P < 0.001 and ^{ns} (not significant).

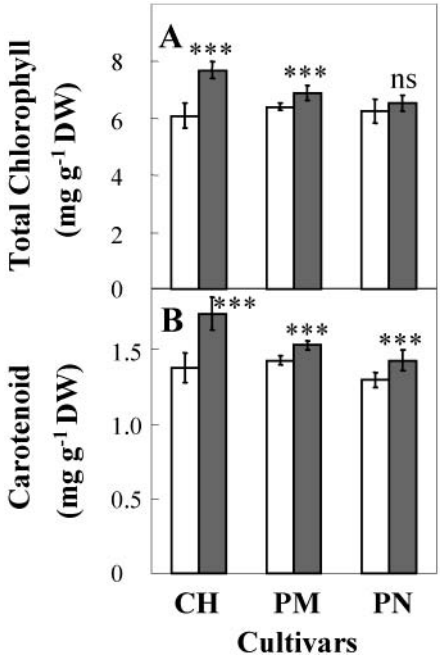

Figure 2. Influence of fmx on photosynthetic pigment concentrations in the vineyard. (A) Chlorophyll concentrations. (B) Carotenoid concentrations. The control and treated plants were represented in white and gray, respectively. CH, Chardonnay; PM, Pinot Meunier; PN, Pinot noir. Data are means ± SE (n = 10). Levels of significance were represented by * at P < 0.05, ** at P < 0.01, *** at P < 0.001 and ^{ns} (not significant).

at 5 and 10 mM fmx. Surprisingly, in vineyard-grown plants, the herbicide treatment increased the leaf chlorophyll content in the three tested cvs., although the difference was not significant for the PN cv. (**Figure 2A**). The highest pigment accumulation was recorded in CH, reaching 126% of the control value.

No significant difference was found in the leaf carotenoid concentration between control and treated cuttings during the first 2 weeks of the experiment (**Table 2**). During the third week, the carotenoid level strongly decreased, by 38 and 63% at 5 and 10 mM fmx, respectively. Conversely, in plants grown in the vineyard, the leaf carotenoid concentrations of the 3 tested cvs. was significantly higher upon fmx exposure (**Figure 2B**).

Carbohydrates. The fluctuations of leaf carbohydrates following fmx treatment were opposite in the two studied models. In cuttings, leaf glucose concentration was transiently lower than

Table 3. Influence of fmx on Glucose, Fructose, Sucrose, and Starch Contents in Fruiting Cuttings

fmx treatment (mM)	content:[a] at days of treatment			
	0	7	14	21
		Glucose Content		
0 (control)	43.1 ± 3.0	56.6 ± 1.2	29.2 ± 0.2	30.0 ± 2.2
0.1		40.3 ± 2.0***	28.6 ± 0.3^{ns}	33.4 ± 1.8^{ns}
5		47.4 ± 3.4**	35.3 ± 1.4**	29.2 ± 2.3^{ns}
10		40.6 ± 0.2***	35.2 ± 0.2**	31.8 ± 3.7^{ns}
		Fructose Content		
0 (control)	42.0 ± 2.6	50.5 ± 0.2	32.6 ± 0.6	27.8 ± 2.1
0.1		35.6 ± 1.5***	31.3 ± 2.5^{ns}	30.4 ± 1.5^{ns}
5		42.3 ± 1.0***	31.9 ± 1.4^{ns}	26.0 ± 0.6^{ns}
10		35.2 ± 1.1***	32.7 ± 0.6^{ns}	21.4 ± 0.8*
		Sucrose Content		
0 (control)	0.50 ± 0.30	0.53 ± 0.34	0.75 ± 0.54	1.10 ± 0.36
0.1		0.57 ± 0.34^{ns}	0.88 ± 0.52^{ns}	1.25 ± 0.69^{ns}
5		2.52 ± 0.56**	3.89 ± 0.88**	0.53 ± 0.28^{ns}
10		1.19 ± 0.35^{ns}	1.13 ± 0.54^{ns}	2.89 ± 0.56**
		Starch Content		
0 (control)	5.40 ± 0.90	5.80 ± 0.51	5.91 ± 0.49	4.52 ± 0.51
0.1		6.24 ± 0.49^{ns}	7.67 ± 1.42^{ns}	4.26 ± 0.07^{ns}
5		7.71 ± 0.52*	8.50 ± 0.62*	4.35 ± 0.72^{ns}
10		7.72 ± 0.51*	6.31 ± 0.91^{ns}	4.58 ± 0.40^{ns}

[a] Results were expressed in mg g^{-1} DW. Data are means ± SE (n = 10). Levels of significance were represented by * at P < 0.05, ** at P < 0.01, *** at P < 0.001 and ^{ns} (not significant).

the control after 1 week of treatment (**Table 3**), but little difference between treatments was noticed thereafter. In contrast, in the vineyard, leaf glucose significantly accumulated in the leaves of fmx-treated plants, by 30, 25, and 15% in CH, PM, and PN, respectively (**Figure 3A**).

Similarly, fructose concentration transiently decreased in the leaves of vine cuttings during the 1st week after fmx application, but no significant difference could be detected afterwards (**Table 3**). However, at the highest fmx concentration (10 mM), fructose level was 23% lower than the untreated plant at the end of the experiment. In vineyard plants, the leaf fructose concentration was significantly increased in the treated plants, by 37, 27, and 13% in the leaves of CH, PM, and PN, respectively (**Figure 3B**).

In both models, the sucrose variation was inverse to that of glucose and fructose. In cuttings, the leaf concentrations of sucrose were much lower than that of glucose or fructose (**Table 3**). No significant difference was observed between the leaf sucrose level in the control plants and those treated with 0.1 mM fmx, whereas sucrose accumulated using higher fmx concentrations: it increased by a 5.2 factor during the 2nd week at 5 mM fmx and by a 2.6 factor during the third week at 10 mM fmx. Conversely, in vineyard, the leaf sucrose concentration was significantly lower in the treated vines whatever the tested cv. (**Figure 3C**). CH was the most sensitive cv., bearing 66% reduction of sucrose content against 14 and 32% in PM and PN, respectively.

In the fruiting cuttings, starch was transiently accumulated during the first week following fmx application at high concentrations (**Table 3**) and then progressively decreased down to values close to the control during the last 2 weeks of treatment. In vineyard-grown plants, responses to fmx treatment fluctuated according to the tested cv. (**Figure 3D**): the starch content increased by 16% in PN, whereas it decreased by 8% in both CH and PM though the difference was not significant in the latter case.

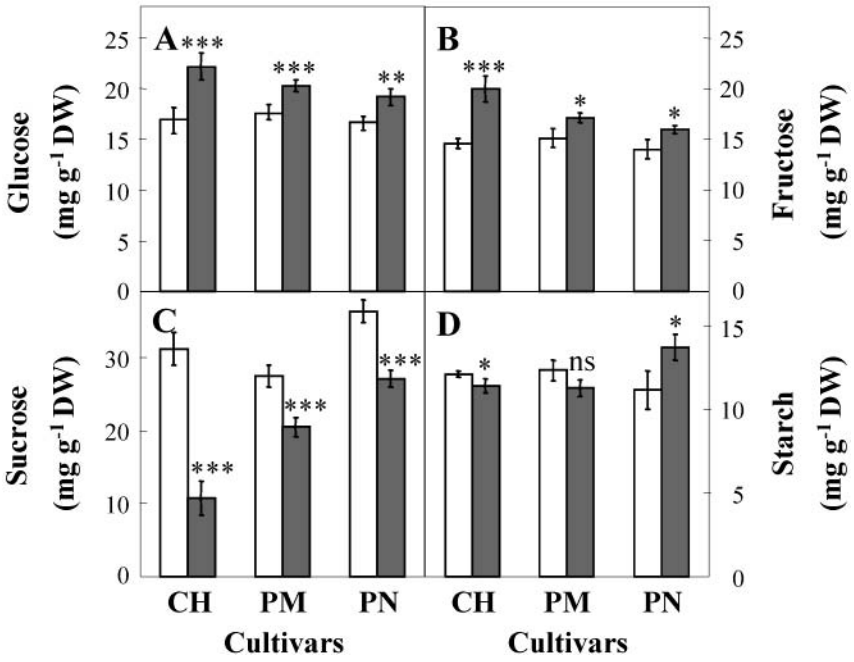

Figure 3. Influence of fmx on leaf carbohydrate contents in the vineyard. (A) Glucose contents. (B) Fructose contents. (C) Sucrose contents. (D) Starch contents. The control and treated plants were represented in white and gray, respectively. CH, Chardonnay; PM, Pinot Meunier; PN, Pinot noir. Data are means ± SE ($n = 10$). Levels of significance were represented by * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$ and ^{ns} (not significant).

DISCUSSION

The results show that the fmx herbicide significantly affects carbohydrate physiology in grapevine using both cuttings and vineyard-grown plants, as reflected by variations in gas exchange, photosynthetic pigment and carbohydrate levels. However, the fmx effects reported in the greenhouse on fruiting cuttings were opposite to those registered in the field. Indeed, fmx caused an alteration of carbon metabolism in fruiting cuttings, in agreement with the effects observed previously on in vitro grown grapevines (*22*). In contrast, for plants grown in vineyard, fmx stimulated photosynthesis and related parameters, though it was applied 5 months earlier during the winter when the annual organs are not yet developed. This suggests that fmx still has residual influence after the development of both the vegetative and reproductive structure. These remnant effects could be due to residual fmx molecules (or derived products) in the soil absorbed in the plant via root uptake (*21*) where they cause a stimulation of plant photosynthesis.

The results indicate that fruiting cutting may not be a good model to evaluate the impact of chemical treatments on field-grown grapevines. Indeed, several parameters differ between these two models. On one hand, fruiting cuttings have no roots when placed in pots. Then, the root system progressively develops during the cutting growth, reducing the barrier to fmx uptake. This may result in a fmx translocation to the leaves and/or transiently limit the herbicide detoxification. We previously showed that fmx is active in photosynthetic tissues of the in vitro grown grapevine via root uptake (*21*, *22*), which may also be the case in cuttings. On the other hand, the soil composition for the cuttings was commercial humus, which may have neither the same adsorption characteristics nor the same soil decomposition activity by microbes that exist in vineyard soil. This may result in greater penetration of the herbicide and thus a stronger herbicide concentration in contact with the cutting root system, generating more toxic effects. Similarly, differences between greenhouse- and field-grown plants were reported on *Ziziphus mauritanita* L. in response to drought (*28*).

In fruiting cuttings, the herbicide flumioxazin had negative consequences on photosynthesis activity. These results are in agreement with the herbicide target, protoporphyrinogen IX oxidase, an enzyme involved in chlorophyll synthesis (*15*). In addition, fmx caused a subsequent decrease in the carotenoid levels. Under light conditions, it has been shown that photobleaching herbicides cause an overproduction of excited chlorophyll molecules, which results in the generation of singlet oxygen (*29*). In turn, this oxidative stress is responsible for carotenoid oxidation and, therefore, the observed decrease of carotenoid concentrations, as reported in other species (*30*, *31*).

Fmx caused a transient accumulation of carbohydrate reserves in the leaves of cuttings, in parallel with a decline of hexoses, suggesting that the cuttings reacted to the herbicide stress by storing sugars in leaves. The accumulation of sugar reserves under stress conditions such as chilling, herbicide treatment, or pathogen infection may be due to an inhibition of the short distance transport of photoassimilates (*32*−*34*). Since sucrose is the main form of transported carbohydrate in grapevine (*35*), the reported sucrose accumulation in mature leaves upon treatment suggests a reduction of carbohydrate export to the sink organs such as growing leaves, roots, and berries.

Amazingly, fmx causes opposite effects in field-grown grapevines, since photosynthesis is still stimulated in the 3 tested cvs. 5 months after spraying. However, the intensity of responses depends on the cv.: it is stronger in CH than in the Pinot cvs. This result is not surprising because the photosynthetic rate is known to vary in different grapevine cvs. (*36*, *37*), and the intensity of responses after fmx exposure may thus differ according to the cv.

Monosaccharides accumulated in mature leaves, whereas sucrose and starch concentrations were lower. This suggests that treated vines may synthesize higher amounts of hexoses, which may be then converted into sucrose and exported to the sink organs. Indeed, at the fruit set stage, young berries and immature leaves, although photosynthetically competent, do not

produce enough carbohydrates to ensure their own growth and remain strong sinks for carbohydrates from mature leaves (*38*, *39*).

In vineyards, 5 months after treatment, there is still an effective impact of fmx on the grapevine leaves that may affect the whole plant physiology. However, the results presented in this work have to be confirmed by studying the carbohydrate translocation in the sink organs, especially regarding flower development and fruit formation. Besides, another remaining question is the effect of fmx or its residues on berry growth and wine quality, since several pesticide residues have been identified in grape berries, affecting both the wine fermentation process (*40*, *41*) and possibly human health.

ABBREVIATIONS USED

CH, Chardonnay; fmx, flumioxazin; PM, Pinot Meunier; PN, Pinot noir.

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