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ORIGINAL PAPER

Arbuscular mycorrhiza partially protect chicory roots against oxidative stress induced by two fungicides, fenpropimorph and fenhexamid

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Abstract The present work examined the oxidative stress induced by different concentrations (0.02 and 0.2 mg l-1) of two sterol biosynthesis inhibitor fungicides (fenpropimorph and fenhexamid) in non-target chicory root colonised or not by *Glomus intraradices* in a monoxenic system. The fungicides were found to cause oxidative damage by increasing lipid peroxidation measured by malondialdehyde production in non-colonised roots. Detoxification of the H_2O_2 product was measured at 0.2 mg l-1 of fenpropimorph by an increase in peroxidase activities suggesting an antioxidant capacity in these roots. Moreover, this study pointed out the ability of arbuscular mycorrhiza to alleviate partially the oxidative stress in chicory roots, probably by lowering reactive oxygen species concentrations, resulting from increases in antioxidant defences. Our results suggest that the enhanced fungicide tolerance in the AM symbiosis could be related to less cell membrane damage.

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Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous in terrestrial ecosystems, forming symbiotic associations with roots of about 70% of the vascular plants (Hodge 2000). Previous studies have shown that AM fungi can alleviate the side effects induced by salt stress, drought, metal pollution, polycyclic aromatic hydrocarbons (PAH), or fungicides (Schützendübel and Polle 2002; Marin et al. 2002; Wu et al. 2006a, b; Zhang et al. 2006; He et al. 2007; Hildebrandt et al. 2007; Debiane et al. 2008).

Sterol biosynthesis inhibitor (SBI) fungicides are the leading group used in agriculture for effective control of plant diseases causing yield reductions in the crops (Hewitt 1998). Among them, the morpholines act on Δ^{14}-reduction and/or $\Delta^8 \rightarrow \Delta^7$ isomerisation (e.g., fenpropimorph) and have been greatly used for the control of pathogenic fungi like powdery mildew, rusts, and leaf blotch diseases in crops. The effects of fenpropimorph on target and non-target organisms have been investigated. Debieu et al. (1992) suggest that fenpropimorph fungitoxicity could be due to the inhibition of sterol target enzymes. Fenpropimorph has also phytotoxic impacts on plants such as growth inhibition, necrotic lesions, and accumulation of abnormal sterols (Grandmougin et al. 1989; Khalil and Mercer 1991; He et al. 2003; Schrick et al. 2004; Campagnac et al. 2008). The side effects of fenpropimorph on several soil organisms have also been detected for soil protozoa (Ekelund 1999; Thirup et al. 2001), saprotrophic

fungi (Bjørnlund et al. 2000), and decomposer fungi in soil (Thirup et al. 2001). Concerning the effects of SBI fungicides on AM fungi, many studies have been conducted but have given contradictory results on fungal development and AM functioning (Sancholle et al. 2001). Using a monoxenic culture system, extraradical development of the AM fungus, *Glomus intraradices*, in contact with fenpropimorph, was found to be strongly affected (Zocco et al. 2008). After application of this fungicide, the mycorrhizal carrot root growth and mycorrhiza colonisation level were also drastically reduced. Modifications in the carrot root sterol composition caused by fenpropimorph could explain the high fenpropimorph toxicity to the AM symbiosis (Campagnac et al. 2008).

Another SBI fungicide group, hydroxyanilide, acts on C4 demethylation (e.g., fenhexamid) and is highly effective against *Botrytis* and related fungal pathogens (Rosslenbroich 1999). Few studies have tested the effects of this fungicide on non-target organisms. Mosleh et al. (2005) showed a negative impact of fenhexamid on the growth of worms. Recently, Zocco et al. (2008) demonstrated a fungistatic effect of high concentrations of fenhexamid on *G. intraradices* spore germination, germ tube elongation, and spore formation. The total colonisation of transformed carrot roots was reported to be not affected, but arbuscule frequency was decreased. The absence of sterol modifications in the roots treated with fenhexamid could account for its more limited impact on mycorrhization (Campagnac et al. 2008).

Many abiotic stresses are accompanied by an enhanced rate of reactive oxygen species (ROS) production, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and alkoxy radical (RO) production. Although they can act as signal molecules, ROS are toxic and can induce cellular damage (Mittler 2002). Thus, high levels of ROS can lead to phytotoxicity (Dat et al. 2000; Parent et al. 2008). Fortunately, plants have the capacity to cope with these ROS by using several antioxidant enzymes and metabolites located in different plant cell compartments (Dat et al. 2000). Several classes of fungicides have been shown to induce plants to increase antioxidant levels in order to protect their cells from oxidative damage by scavenging ROS (Wu and von Tiedemann 2001; Jaleel et al. 2006; Gopi et al. 2007; Zhang et al. 2007). The superoxide radical ($O_2^{\cdot-}$) is usually catalysed by superoxide dismutases (SODs) to H_2O_2, a product relatively stable but toxic. Catalases and peroxidases (PODs) remove the bulk of H_2O_2 (Van Breusegem et al. 2001). Nevertheless, antioxidant capacity is dependent on the severity of the stress, on the species, and/or on the stage of development (Dat et al. 2000). Excessive level of ROS can result in cell damage involving lipid oxidation of cell membranes (Dotan et al. 2004).

The plasma membrane has been suggested to be an efficient barrier preventing some pollutants from entering the symplasm (Taylor 1988). As a response to environmental stresses, cells can modify their membrane lipid composition in order to maintain optimal physical properties (Thompson 1992). The regulation of the lipid composition and the adjustment of the unsaturation level of membrane fatty acids (FA) are very important to deal with pollutant toxicity and make the plant tolerant so as to survive in unfavourable conditions (Thompson 1992; Bidar et al. 2008). Alteration in membrane phospholipids caused by lipid peroxidation is a common criterion of oxidative stress (Halliwel and Gutteridge 1989). Polyunsaturated FA decomposition leads mainly to the production of malondialdehyde (MDA), which reflects the level of membrane peroxidation (Dotan et al. 2004). Another parameter to determine the oxidative stress is the 8-hydroxy-2′-deoxyguanosine (8-OHdG), which has been studied as a marker for oxidative DNA damage (Dotan et al. 2004).

In the last decade, many studies on AM fungi have been carried out with monoxenic culture systems. This technique has improved the understanding of the symbiosis (Declerck et al. 2005). Monoxenic cultures enable non-destructive observations of AM fungi (Fortin et al. 2002); they are standardised and provide a reproducible system to compare the effects of different molecules on AM fungi (Debiane et al. 2008; Hillis et al. 2008; Zocco et al. 2008). Furthermore, a large quantity of biological material free of contaminant microorganisms, which contain lipids and different enzymatic activities, can be obtained using these cultures.

In the present study, we used monoxenic cultures in order to link the direct impact of two SBI fungicides (fenpropimorph and fenhexamid) on AM symbiosis development (chicory roots/*G. intraradices*) with oxidative stress, by analysing FA composition and by measuring MDA production, SOD- and POD-specific activities, and 8-OHdG formation. Results provide information firstly, about the oxidative damage caused by the two SBI fungicides tested and secondly, about the ability of mycorrhiza to partially alleviate the oxidative stress in chicory roots.

Materials and methods

Plant and fungal material

Ri T-DNA-transformed chicory roots (*Cichorium intybus* L.), colonised or not by *G. intraradices* Schenck and Smith (DAOM 197198), were grown on modified Strullu–Romand (MSR) medium (Strullu and Romand 1986, modified by Declerck et al. 1998) solidified with 0.25% (*w*/*v*) gellan gel (Phytagel: Sigma, St Louis, MO, USA) at 27°C in the dark. Medium was inoculated with standardised root inoculum of

2-month-old monoxenic cultures of Ri T-DNA-transformed chicory roots, colonised or not by *G. intraradices*, using a 10-mm cork borer as described by Verdin et al. (2006). A disc of culture medium containing roots from monoxenic cultures (non-colonised or colonised chicory roots) was placed in the middle of each Petri dish containing the medium without (control) or with the different concentrations of the two fungicides tested (fenpropimorph and fenhexamid). After 9 weeks of culture, roots used as inoculum were discarded and were not taken into account in the biomass estimation and in the different parameters analysed.

SBI fungicide treatments

Fenpropimorph and fenhexamid (technical grade) were supplied by Baden Aniline and Soda Factory (BASF) AG (Germany) and Bayer Crop Science (Germany), respectively. For each experiment, fungicides were dissolved in acetone (0.5 ml l^{-1} medium) and added to sterilised (121°C for 15 min) MSR medium (80°C) in order to obtain final concentrations of 0.02 and 0.2 mg l^{-1} of SBI fungicides. The bottles were agitated by hand, and a 25-ml aliquot of MSR medium was poured into each standard Petri dish (9 cm). The control contained MSR medium without SBI fungicide and received the same quantity of acetone as all treatments. Zohrehvand (2005) showed that the most of this solvent evaporated on contact with the MSR medium. Moreover, no toxic effect was observed for the AM fungus or host root at the concentration used (0.5 ml l^{-1} medium) (Wan et al. 1998; Zocco et al. 2008; Campagnac et al. 2008).

Determination of *G. intraradices* development and chicory root colonisation

After 9 weeks of culture in the dark at 27°C, the length of the hyphae was measured by the gridline method, and the data were integrated using the formula of Tennant (1975). Number of spores was counted using the method described by Declerck et al. (2001).

To collect roots, root culture media were solubilised for 10 min under agitation in 1 vol Tris buffer (Tris HCl 50 mM, pH 7.5, ethylenediaminetetraacetic acid 10 mM) at room temperature and collected by filtration on a 0.5-mm sieve. Roots were rinsed with sterile water and frozen at −80°C. One part of root samples was cleared in KOH and stained with Chlorazol black E (Brundrett et al. 1994) to quantify *G. intraradices* colonisation using the gridline intersect method (McGonigle et al. 1990).

Preparation of crude cell-free extracts

A second part of the collected root samples was used to determine MDA production, SOD and POD activities, 8-OHdG formation, and protein contents. Frozen tissues from roots samples were ground using the grinding Precellys 24 (Bertin Technologies, Montiguy-Le-Bretonneux, Yvelines). Samples were then suspended in 1 ml of phosphate buffered saline (10 mM). After centrifugation (3 min/10,000 g), supernatants were divided in 250-μL aliquots to determine the parameters previously cited.

Determination of MDA concentration

A high-performance liquid chromatography (HPLC) assay was used to evaluate MDA production (Shirali et al. 1994). Two hundred microlitres of root aliquot were mixed with 1 ml of 0.1 N HCl and extracted twice with 3 ml of ethyl acetate. The mixture was shaken for 5 min and centrifuged at 3,000 g for 10 min. The organic layers were collected and evaporated under a stream of nitrogen. After evaporation, the extract was suspended in 100 μL of methanol. The HPLC system of Jasco PU-980 pump was equipped with a Nucleosil column (C18, 150 × 4.6 mm, 5 μm particle size), a Rheodyne 7725 automated injector, a UV detector (detection wavelength=532 nm), and a Shimadzu CR3A integrator (Vasse Industries, Lille, France). The mobile phase was a blend of 50 mM KH_2PO_4 and methanol 60/40 (*v*/*v*) adjusted to pH 6.8 (KOH 1 M). Tetraethoxypropane (Sigma, Saint Quentin Fallavier, France) was used as the standard, and thiobarbituric acid (TBA) as the reagent for MDA assay. One hundred microlitres of either standard solutions or methanol extracts were injected in the HPLC system, and the MDA-TBA adducts were detected.

Determination of SOD and POD activity

Total SOD and POD activities were measured in supernatants using a commercially available reagent kit (Superoxide Dismutase Kit, R&D Systems Europe, Abingdon, UK) for the SOD assay and the method described by Mitchell et al. (1994) for the POD assay.

Determination of 8-OHdG formation

The 8-OHdG enzyme-linked immunosorbent assay (ELISA) kit used gives a competitive immunoassay for quantitative measurement of the oxidative DNA adducts 8-OHdG (8-OHdG ELISA; Japan Institute for the Control of Aging, Haruoka, Japan). DNA was extracted using DNeasy™ Plant Mini Kit (Qiagen, Courtaboeuf, France), and the DNA concentration was determined by spectrophotometry from the absorbance at 260 nm (GeneQuant II RNA/DNA Calculator, Pharmacia BioTech Europe, Orsay, France). Thirty-eight microlitres of DNA suspension was incubated at 100°C for 2 min, treated with 3 μl of 250 mM potassium acetate buffer (pH 5.4), 3 μl of 10 mM zinc

sulphate, and 2 µl of nuclease P1 (6.25 U/µl; Sigma-Aldrich) at 37°C overnight, and then treated with 6 µl of 0.5 M Tris–HCl (pH 8.2) and 2 µl of alkaline phosphatase (0.31 U/µl; Sigma-Aldrich), at 37°C for 2 h.

Determination of total protein concentrations

Total protein concentrations were determined in root extracts with the bicinchoninic reagent using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Saint Quentin Fallavier, France).

Fatty acid extraction, analysis, and identification

Before FA extraction, chicory roots were lyophilized during 48 h. The freeze-dried plant material (15 mg dry weight) was saponified with 3 ml of 6% (*w*/*v*) KOH in methanol at 85°C for 2 h. After extraction of the unsaponiable fraction with hexane, the saponifiable fraction was adjusted to pH 1 with HCl (6 N). FA, after addition of 1 vol of distilled water, were extracted (×3) with 5 vol of hexane and evaporated under N_2. FA were methylated using 3 ml of BF3/methanol (14%) at 70°C for 3 min, and reaction was stopped in ice (Morrison and Smith 1964). The FA methyl esters were extracted (×3) with 5 vol of hexane after the addition of 1 ml of distilled water. These extracts were evaporated under N_2 and transferred to chromatography vials.

Final extracts were analysed using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionisation detector (Norwalk, CT, USA) and a EC-1000 (Alltech) capillary column (30 m × 0.53 mm i.d.) with hydrogen as carrier gas (3.6 ml min^{-1}). The temperature programme included a fast rise from 50°C to 150°C at 15°C min^{-1} and then a rise from 150°C to 220°C at 5°C min^{-1}. FA were quantified using heptadecanoic acid methyl ester (C17:0) as an internal standard and by introducing a defined amount of this compound into every sample just before running on GC. Their identification relied on the retention times of a wide range of standards. Overall, 37 different reference FAs were used as standards (lipids standards: FA methyl ester mixtures C4–C24:1, Sigma-Aldrich).

Statistical analysis

ANOVA analysis was carried out with the statistical programme Statgraphics release 5.1 (Manugistic, Inc. Rockville, MD, USA). The method used to discriminate between the means was the least significant difference test ($P<0.05$). Analysis of variance was conducted on the original data except for the data of root colonisation percentage and data of FA percentage, which was converted to arcsine values before the analysis of variance. Levene's test of variance homogeneity was checked before the use of the multiple comparison procedure. The method used to compare non-mycorrhizal and mycorrhizal roots for a same treatment was the Student test ($P<0.05$).

Heterogeneous variance was detected with data on hyphal length and sporulation. A non-parametric test as Kruskal–Wallis for trend was applied. The differences between the treatments were estimated by the Siegel–Castellan test of multiple comparisons (Siegel and Castellan 1988). Test utility application of Kruskal–Wallis was developed by Georgin and Gouet (2005) and test utility application of multiple comparisons by Le Pape based on Siegel and Castellan (1988). All the tests were performed with five replicates per treatments.

Results

SBI fungicide effects on the chicory root fatty acid composition

The FA composition of transformed chicory roots incubated in the absence and presence of different concentrations of fenpropimorph and fenhexamid (0.02 and 0.2 mg l^{-1}), colonised or not by *G. intraradices*, is presented in Table 1. The FA composition of non-mycorrhizal roots ranged from C16:0 to C18:3. The predominant FA compounds were C16:0 (palmitic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid); they constituted more than 93% of the total FA. C18:2 was the major compound (54%). The FA profile as well as the total FA contents remained unchanged after treatment with either SBI fungicide as compared to the control, but FA proportions and quantities were affected by the presence of 0.2 mg l^{-1} of fenpropimorph: C16:0 significantly increased, whilst C18:3 decreased as compared to the control.

The FA composition of mycorrhizal roots differed from non-mycorrhizal ones by the presence of C16:1ω5, which is a major FA for most *Glomus* isolates (Beilby 1980; Nordby et al. 1981; Pacovsky and Fuller 1988; Graham et al. 1995; Grandmougin-Ferjani et al. 2005). It represents 20% of the total FA. The total FA contents were not modified by SBI fungicide exposure. In contrast, the relative proportions and quantities of some FA were affected by the presence of fenpropimorph. Indeed, the AM fungal marker C16:1ω5 (Olsson 1999) significantly decreased by 40% and 64% at 0.02 and 0.2 mg l^{-1} of fenpropimorph, respectively, as compared to the untreated roots.

The ratio of saturated/unsaturated FA significantly increased by 18% and 20% at 0.2 mg l^{-1} of fenpropimorph in non-mycorrhizal and mycorrhizal roots, respectively.

Table 1 Fatty acid composition of transformed non-mycorrhizal and mycorrhizal chicory roots (*Cichorium intybus* L.) after 9 weeks of growth in the absence or presence of fenpropimorph and fenhexamid

Non-mycorrhizal roots (fungicides; mg l^{-1})	Control		Fenpropimorph				Fenhexamid			
			0.02		0.2		0.02		0.2	
Fatty acids	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%
C16:0	2.04±0.11a	22a	2.00±0.08a[a]	21a	2.75±0.26c	25b	1.83±0.12ab	21a	1.78±0.08ab	21a
C16:1ω5	–	–		–		–		–		–
C18:0	0.26±0.01ab	3a	0.25±0.01b	3a	0.34±0.04a	3a	0.26±0.02b	3a	0.23±0.01b	3a
C18:1	0.34±0.05a	4a	0.28±0.06a	3a	0.34±0.01a	3a	0.37±0.11a	4a	0.26±0.03a	3a
C18:2	5.37±0.13a	54a	5.56±0.33ab	59a	6.69±0.72b	60a	5.17±0.31a	59a	5.04±0.26a	59a
C18:3	1.33±0.15a	18a	1.28±0.08ab	14a	0.95±0.14b	8b	1.16± 0.08ab	13a	1.18±0.08ab	14a
Total FA (mg g^{-1} dry weight)	9.33±0.46a		9.37±0.54a		11.07±1.17a		8.78±0.63a		8.48±0.46a	
Saturated/unsaturated FA	0.33a		0.32a		0.39b		0.31a		0.31a	
Mycorrhizal roots (fungicides; mg l^{-1})	**Control**		**Fenpropimorph**				**Fenhexamid**			
			0.02		0.2		0.02		0.2	
Fatty acids	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%
C16:0	2.28±0.27a[a]	21ab	2.54±0.11a[a]	21b	2.66±0.25a	24c	2.36±0.34a	20a	2.38±0.27a[a]	21ab
C16:1ω5	1.90±0.12a	20a	1.48 ±0.31b	12bc	0.78±0.09c	7c	1.82±0.43a	16ab	2.28±0.55a	20a
C18:0	0.25±0.02a	2a	0.32±0.03a	3a	0.31±0.04a	3a	0.27±0.04a	2a[a]	0.25±0.01a	2a
C18:1	0.25±0.12a	3a	0.40±0.04b	3a	0.48±0.05b	4a	0.42±0.07b	4a	0.46±0.06b	4a
C18:2	5.00±0.74a	45a[a]	6.09±0.26b	51ab[a]	5.86±0.59b	54b[a]	5.60±0.79ab	48a[a]	5.09±0.62a	44a[a]
C18:3	1.04±0.12ab	9a[a]	1.21±0.05a	10a[a]	0.92±0.17b	8a	1.17±0.13ab	10a[a]	1.04±0.09ab	9a[a]
Total FA (mg g^{-1} dry weight)	10.72±1.17a		12.04±0.79a[a]		11.02±1.35a		11.65±1.68a[a]		11.50±1.31a[a]	
Saturated/unsaturated FA	0.30a		0.31a		0.36b		0.30a		0.30a	

Data are presented as means ± standard error). The means were obtained from five replicates

Different letters on a same line (a fatty acid) indicate significant differences ($P<0.05$) between different concentration treatments according to the least significant difference test

[a] Indicates significant difference between non-mycorrhizal and mycorrhizal roots according to the least significant difference test ($P<0.05$)

– Not detected

When FA compositions of non-colonised roots and colonised roots are compared, no significant modifications in the quantities are observed. The changes detected in the relative amounts of some FA are due to the presence of C16:1ω5 in mycorrhizal roots.

SBI fungicide effects on oxidative stress parameters

MDA contents measured in non-mycorrhizal and mycorrhizal roots in the absence and presence of the two SBI fungicides are presented in Table 2. In non-colonised roots, significant increases in MDA contents were observed both in the presence of fenpropimorph (0.02 and 0.2 mg l^{-1}) and fenhexamid (0.02 mg l^{-1}) as compared to the control. In contrast, in mycorrhizal roots, whatever the treatment, MDA contents were constant and similar to the control. Moreover, MDA production was significantly lower in colonised roots than in non-colonised roots except in the presence of 0.2 mg l^{-1} of fenhexamid (Table 2).

No significant changes in SOD activities were found in mycorrhizal or non-mycorrhizal roots in the presence of the tested SBI fungicides in comparison to their controls grown without fungicide (Table 2). In addition, SOD activities were constant between AM roots versus non-AM roots.

No significant increase in POD activities was detected in non-mycorrhizal roots when compared to the control without fungicides, except with 0.2 mg l^{-1} of fenpropimorph. On the contrary, in mycorrhizal roots, increments in POD activities were observed in the presence of fenpropi-

Table 2 Concentration of malondialdehyde (MDA), superoxide dismutase (SOD), and peroxidase (POD) activities and 8-hydroxy-2-desoxyguanosine (8-OHdG) in transformed chicory roots (*Cichorium intybus* L.) colonised or not by *Glomus intraradices* (mycorrhizal roots (M) or non-mycorrhizal roots (NM)) after 9 weeks of growth in the absence or presence of fenpropimorph and fenhexamid

Fungicides (mgl^{-1})	MDA (µmol/µg of protein)		SOD activity (U/g of protein)		POD activity (U/g of protein)		8-OHdG (ng/µg of DNA)	
	NM	M	NM	M	NM	M	NM	M
Control	0.124±0.022a	0.063±0.008a[a]	10.186±1.051a	10.734±0.375a	0.047±0.023ab	0.022±0.014a	5.185±0.364a	1.248±0.764a[a]
Fenpropimorph 0.02	0.203±0.017b	0.057±0.003a[a]	10.013±0.887a	9.830±0.875a	0.009±0.001a	0.071±0.007ab[a]	3.080±1.815a	1.813±0.181a[a]
Fenpropimorph 0.2	0.281 ±0.022c	0.088±0.013a[a]	9.920±0.644a	11.001±1.277a	0.304±0.032c	0.216±0.025c	4.083±0.406a	1.628±0.516a[a]
Fenhexamid 0.02	0.223±0.013bc	0.056±0.008a[a]	9.480±1.219a	12.281±0.589a	0.092±0.009b	0.083±0.018b	1.863±0.546a	1.760±0.843a
Fenhexamid 0.2	0.111±0.033a	0.062±0.011a	10.833±1.816a	11.408±0.696a	0.070±0.013b	0.099±0.011b	6.807±2.439 a	3.316±3.316a

Data are presented as means ± SE (n=5). The means were obtained from five replicates

Different letters on a same column indicate significant differences (P< 0.05) between different concentration treatments according to the least significant difference test

[a] Indicates significant difference between non-mycorrhizal and mycorrhizal roots according to the least significant difference test (P<0.05)

morph (0.2 mg l^{-1}) and fenhexamid (0.02 and 0.2 mg l^{-1}) in comparison to their controls without fungicide (Table 1). In addition, no significant differences were detected when POD activities were compared in AM roots versus non-AM roots, except with 0.02 mg l^{-1} of fenpropimorph (Table 2).

Table 2 presents results on DNA damage, measured by 8-OHdG formation, in mycorrhizal and non-mycorrhizal chicory roots treated with the SBI fungicides. The oxidative DNA adducts 8-OHdG were detected both in mycorrhizal and non-mycorrhizal roots in the presence and absence of fungicides. However, significant differences were detected in the 8-OHdG concentrations when comparing colonised and non-colonised roots exposed to fenpropimorph and in the control without fungicide (Table 2). A reduction in the amounts of 8-OHdG was observed in mycorrhizal roots grown without fungicide and treated with fenpropimorph, when compared to the non-colonised roots.

SBI fungicide effects on chicory root colonisation and fungal development

The chicory root colonisation by *G. intraradices* after 9 weeks of incubation in the absence and presence of the two SBI fungicides is shown in Fig. 1a. The percentage of mycorrhization in chicory roots grown without fungicide (control) reached 25% for hyphae, 5% for vesicles, and 10% for arbuscules. The hyphal colonisation of the chicory roots was significantly reduced by fenpropimorph treatments as compared to the control. Only 11% and 2% of colonisation were obtained with 0.02 and 0.2 mg l^{-1} of fenpropimorph, respectively. The vesicles were significantly reduced as compared to the control only in the presence of 0.2 mg l^{-1} fenpropimorph. No arbuscules were observed in the presence of fenpropimorph whatever the concentration tested. In contrast, no significant impact on colonisation (hyphae, vesicles, and arbuscules) was observed with the fenhexamid treatment as compared to the non-treated control.

The number of spores produced by the colonised roots grown in the absence and presence of different SBI fungicide concentrations after 9 weeks of culture is presented in Fig. 1b. The number of spores produced in the non-treated cultures was about 350. Significant decreases in sporulation occurred following fenpropimorph exposure. Whereas spore formation was reduced by 2.5-fold in the presence of 0.02 mg l^{-1} of fenpropimorph, it was completely inhibited at 0.2 mg l^{-1}. With fenhexamid, the reduction in sporulation was less drastic.

The development of extraradical hyphae of *G. intraradices* grown in treated and non-treated roots with the two fungicides is presented in Fig. 1b. The extraradical hyphae length measured in the non-treated cultures reached 435 cm

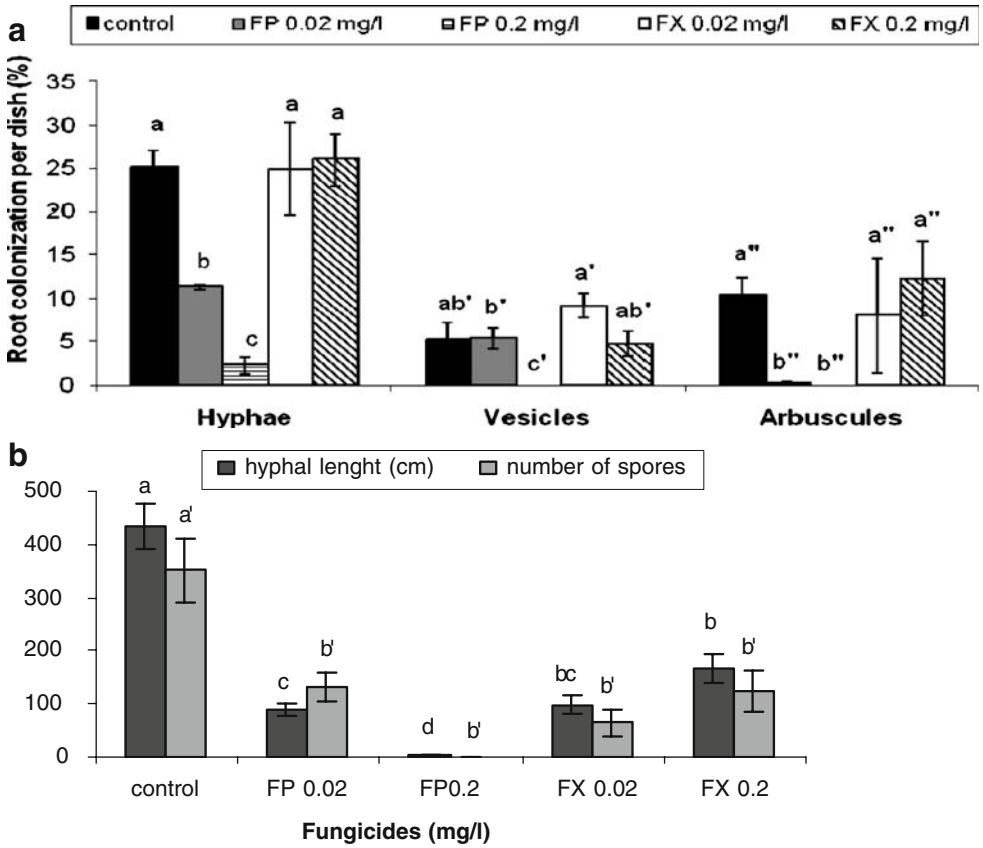

Fig. 1 Effect of different concentrations of fungicides (**a**) on the root colonisation (hyphae, vesicles, and arbuscules), (**b**) on the extraradical hyphae development, and on the spore production of *Glomus intraradices* after 9 weeks of growth in the absence or presence of fenpropimorph (*FP*) and fenhexamid (*FX*). The means were obtained from five replicates. Data of colonisation percentage were converted to arcsine values before the analysis of variance for comparing the means according to the least significant difference test ($P<0.05$). *Different letters* indicate significant differences between different concentration treatments

after 9 weeks of incubation. An important reduction in hyphal length was observed with fenpropimorph treatment as compared to the control. A decrease of 80% occurred at 0.02 mg l^{-1}, and a total inhibition was observed at 0.2 mg l^{-1} of fenpropimorph. With the higher concentration of fenhexamid, the reduction in extraradical hyphae length was less extensive.

SBI fungicide effects on the root growth

SBI fungicide effect on chicory root growth was determined by measuring dry weight after 9 weeks incubation in the absence and presence of fenpropimorph and fenhexamid at 0.02 and 0.2 mg l^{-1}. The results presented in Fig. 2 showed that whereas the root biomass produced in the presence of fenhexamid and 0.02 mg l^{-1} of fenpropimorph was similar to the control, the dry weight obtained at 0.2 mg l^{-1} fenpropimorph was significantly lower as compared to the control without fungicide. The drastic decrease, estimated at about 90%, was observed both in non-colonised and *G. intraradices*-colonised roots.

Discussion

Plants are exposed to various types of environmental xenobiotics, sometimes deliberately as in the case of agricultural pesticides. Several studies have reported phytotoxic effects of morpholine SBI fungicides such as growth reduction, necrotic lesions, and altered phytosterol composition (Costet-Corio and Benveniste 1988; Khalil and Mercer 1991; Schaller et al. 1991; He et al. 2003; Schrick et al. 2004). With fenpropimorph, the toxic effect observed on plant development has been attributed to altered sterol composition, especially accumulation of atypical $\Delta^{8,14}$-sterols (He et al. 2003). Up to present, no study is available on the eventual induction of oxidative stress in plants by this compound and by fenhexamid belonging to another SBI class (hydroxyanilide).

The data reported in the present work point to a differential toxic effect of fenpropimorph and fenhexamid on *G. intraradices*-colonised and non-colonised chicory roots. Whereas root growth was severely affected by 0.2 mg l^{-1} of fenpropimorph, fenhexamid did not exhibit any effect. This result is in agreement with the previous

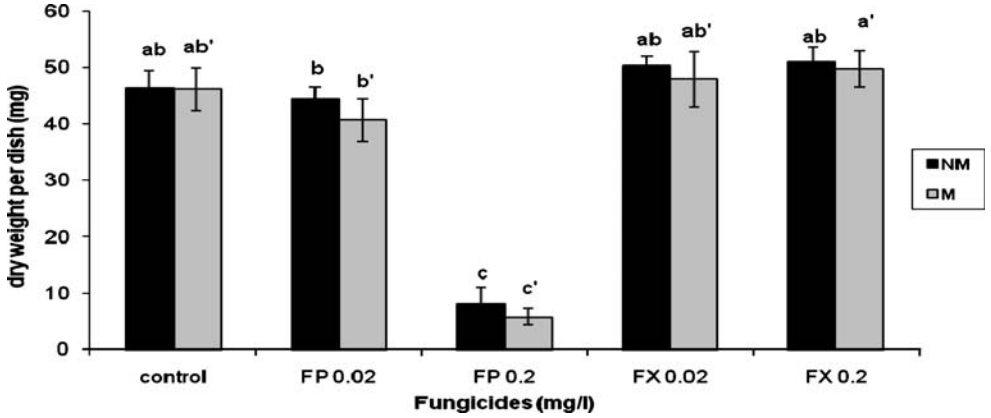

Fig. 2 Biomass of transformed chicory roots (*Cichorium intybus* L.) colonised or not by *Glomus intraradices* (mycorrhizal roots (*M*) or non-mycorrhizal roots (*NM*)) after 9 weeks of growth in the absence or presence of fenpropimorph (*FP*) and fenhexamid (*FX*). Data are presented as mean ± SE. The means were obtained from five replicates; *different letters* indicate significant differences between different concentration treatments; an *asterisk* indicates significant differences between *NM* and *M* roots according to the least significant difference test ($P<0.05$)

studies of Zocco et al. (2008) and Campagnac et al. (2008), who worked with monoxenic cultures of transformed carrot roots. In addition, fenpropimorph induced strong modifications in carrot root sterol composition with an accumulation of unusual compounds such as 9β,19-cyclopropylsterols (24-methylpollinastanol), $\Delta^{8,14}$-sterols (ergosta-8,14-dienol, stigmasta-8,14-dienol), Δ^{8}-sterols (Δ^{8}-sitosterol), and Δ^{7}-sterols (ergosta-7,22-dienol), whereas fenhexamid did not have any effect on root sterol profiles (Campagnac et al. 2008). The important toxicity of fenpropimorph observed for chicory roots could be due to modified sterol composition. Although there is strong evidence that sterols are essential for normal plant growth (He et al. 2003), other mechanisms which strengthen the phytotoxicity observed in fungicide-treated roots cannot be excluded.

In the present work, we have studied alterations in FA composition and content, lipid peroxidation (evaluated in terms of MDA content), and antioxidant enzyme activities (evaluated through the determination of SOD and POD activities) in the chicory roots grown in the presence of fenpropimorph and fenhexamid, in order to highlight other mechanisms which could be involved in SBI fungicide toxicity. This represents the first evaluation of oxidative stress in plants treated by SBI fungicides belonging to morpholine and hydroxyanilide groups, contrary to the many studies concerning the azole group (Wu and von Tiedemann 2001; Gopi et al. 2007; Manivannan et al. 2007; Kishorekumar et al. 2008).

The finding that the non-colonised chicory root FA composition ranged from C16:0 to C18:3, with three major compounds (C16:0, C18:2, and C18:3), is in accordance with FA composition of chicory leaves reported by Blanckaert et al. (2000). The total FA content of the non-colonised chicory roots was similar in the absence or presence of either of the fungicides tested whatever the concentration. In contrast, the FA quantities and proportions were affected by the presence of 0.2 mg l^{-1} of fenpropimorph. Whereas C16:0 significantly increased, C18:3 decreased as compared to the control. Similar observations have been reported in plants exposed to a metallic pollutant (Ouariti et al. 1997; Ben Youssef et al. 2003; Bidar et al. 2008). The reduction in C18:3 by the higher concentration of fenpropimorph suggests an induction of lipid peroxidation. This hypothesis is reinforced by the fact that the MDA content in roots exposed to this fungicide was significantly higher than in the non-treated control. The decrease in C18:3 levels may therefore be related to the direct reaction of oxygen-free radicals with unsaturated lipids. Indeed, one of the most common reactive aldehydes, MDA, results from the oxidative degradation of polyunsaturated FA with more than two methylene-interrupted double bonds (Esterbauer et al. 1991).

Moreover, the increase in the ratio of saturated/unsaturated FA both in non-AM and AM roots exposed to 0.2 mg l^{-1} of fenpropimorph suggests a probable modification in the phospholipids-associated FA. Several authors have reported that pollutants like heavy metals enhanced lipoxygenase activity (Clijsters et al. 1991; Somashekaraiah et al. 1992; Sanità Di Toppi and Gabbrielli 1999; Skórzyńska-Polit and Krupa 2006; Zhou et al. 2008), and several studies have proposed a role of lipoxygenase in the loss of membrane integrity following stress conditions (Siedow 1991). Lipoxygenase can efficiently start peroxidative breakdown of polyunsaturated FA and/or generate ROS, which can increase the deterioration and permeability of membranes (Thompson et al. 1998).

The oxidative stress arising from abiotic stress exposure could generate ROS (Apel and Hirt 2004), which can interact with polyunsaturated FA to generate aldehydes:

among them, MDA is one of the most abundant oxidative by-products and is very often used as a suitable biomarker of lipid peroxidation (Bailly et al. 1996). Recently, MDA was found to be able to modify proteins (Yamauchi et al. 2008) and was supposed to be involved in the deterioration of various biological functions through its attachment to proteins and nucleic acids. In the present work, a significant increase in the MDA content was observed in non-AM roots exposed to fenpropimorph and fenhexamid, except for the lower concentration of fenhexamid tested. These observations are in agreement with other studies reporting that other abiotic stresses, such as PAH, metal pollution, drought, salinity, and heat, induce MDA production by plant cells (Sinha et al. 2005; Bidar et al. 2007; He et al. 2007; Debiane et al. 2008; Yamauchi et al. 2008).

Antioxidative enzymes are important components in preventing oxidative stress in plants. The activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Allen 1995), and they play an important role in ROS catabolism. In the present study, SOD activities in chicory roots remained constant after fungicide treatment suggesting either that this antioxidant enzyme is not implicated in ROS scavenging in chicory roots or that induction occurred before or after the sampling time point. In contrast, activities of POD, another antioxidant enzyme which degrades H_2O_2, were increased at 0.2 mg l^{-1} of fenpropimorph concentration in comparison to non-treated controls, indicating an enhancement of free radicals under this fungicide stress.

Accumulation of ROS in cells treated with pollutants can also lead to oxidative damage of DNA (Escobar et al. 1996). In the present work, DNA alteration evaluated in terms of 8-OHdG DNA adduct formation in fungicide-exposed roots did not show any increase compared to non-treated controls, suggesting an insensitivity of the chicory DNA to the SBI fungicides.

It can be concluded that both of the SBI fungicides tested cause oxidative damage as indicated by the increased lipid peroxidation, measured by MDA production. Detoxification of the H_2O_2 product and increases in POD activities were measured at the higher concentration of fenpropimorph, suggesting the development of an antioxidant capacity in the chicory roots. The relation between fungicide sensitivity and lipid peroxidation in response to fenpropimorph indicates that fenpropimorph toxicity resulted in increased production of ROS. Furthermore, the more toxic effect of 0.2 mg l^{-1} of fenpropimorph as compared to fenhexamid and 0.02 mg l^{-1} of fenpropimorph is consistent with the drastic decrease in growth of the chicory roots in presence of the higher concentration of fenpropimorph.

Whereas fenhexamid was harmless to chicory mycorrhizal root growth and development of *G. intraradices*, AM colonisation was significantly reduced after fenpropimorph treatment (0.02 and 0.2 mg l^{-1}). In contrast, *G. intraradices* development, estimated by extraradical hyphae length and spore formation, was significantly decreased by both fungicides. These observations are in accordance with other studies, which reported the negative effect of various pollutants (i.e., PAH and heavy metals) and other abiotic stresses (i.e., salinity) on the development of *Glomus* sp. (Schützendübel and Polle 2002; Verdin et al. 2006; He et al. 2007; Hildebrandt et al. 2007; Debiane et al. 2008). Whilst previous studies on the effect of the SBI fungicides on mycorrhizal plants have given contradictory results (Sancholle et al. 2001), our observations on the reduced extraradical development of *G. intraradices* are in complete agreement with the studies of Zocco et al. (2008) and Campagnac et al. (2008) on monoxenic-cultured carrot roots treated with fenhexamid and fenpropimorph.

The FA C16:1ω5 (20%) is considered as an AM fungal marker (Olsson 1999). A significant decrease in C16:1ω5 was observed in AM chicory roots after SBI fungicide exposure, whilst the total FA content remained unchanged. This decrease was associated with the negative effect of fenpropimorph on chicory root colonisation. Interestingly, no significant change was observed in the C18:3 proportion or amount in mycorrhizal roots with fungicide application. Indeed, MDA content of mycorrhizal roots did not increase significantly with fungicide treatments, indicating less lipid peroxidation in AM roots as compared to non-AM roots. Moreover, MDA contents were significantly lower in AM roots than in non-AM roots. It seems that cellular damage by free radicals is alleviated partially by mycorrhization, which presumably contributes to fungicide tolerance in AM chicory roots. This protection of root cells against oxidative damage of membranes by ROS in AM roots may be related to the accumulation of apocarotenoids in roots (Strack and Fester 2006). Since POD activities were not more important in AM chicory roots, this enzyme is not responsible for the less ROS produced in AM roots. According to our knowledge, only few data have previously reported a potential role of mycorrhization in reducing lipid peroxidation in plants exposed to pollutants (Debiane et al. 2008).

In conclusion, the results from the present study not only contribute to knowledge about the toxicity of agricultural chemicals for the AM symbiosis but also show that the biological system used could provide a useful approach for soil ecotoxicology studies and risk assessment. They suggest a possible relationship between fungicide toxicity, production of ROS under fungicide stress, lipid peroxidation, and alteration of membranes. In order to better understand plant and symbiosis tolerance to these fungicides in terms of membrane alteration, it could be interesting, in future work, to consider lipid classes that

constitute the membrane (i.e., phospholipids and sterols) and to study FA associated with phospholipids. In this context, a study of lipid rafts, functional microdomains of the plasma membrane, could be particularly informative, especially since it was recently demonstrated that the membrane structure of hepatocytes, depicted as membrane fluidity and lipid rafts, plays a key role in induced oxidative stress (Nourissat et al. 2008). Likewise, it would be interesting to assess lipoxygenase activity in AM roots grown under SBI treatment, considering that MDA is a product of lipid peroxidation, as well as an indicator of free radical production and tissue damage, and that lipoxygenase activity increases as a consequence of increased lipid peroxidation due to pollutant toxicity (Sanità Di Toppi and Gabbrielli 1999 Skórzyńska-Polit and Krupa 2006; Zhou et al. 2008).

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