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ORIGINAL PAPER

Fenpropimorph slows down the sterol pathway and the development of the arbuscular mycorrhizal fungus *Glomus intraradices*

E. Campagnac · J. Fontaine · A. Lounès-Hadj Sahraoui · F. Laruelle · R. Durand · A. Grandmougin-Ferjani

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Abstract The direct impact of fenpropimorph on the sterol biosynthesis pathway of *Glomus intraradices* when extraradical mycelia alone are in contact with the fungicide was investigated using monoxenic cultures. Bi-compartmental Petri plates allowed culture of mycorrhizal chicory roots in a compartment without fenpropimorph and exposure of extraradical hyphae to the presence of increasing concentrations of fenpropimorph (0, 0.02, 0.2, 2, 20 mg l^{-1}). In the fungal compartment, sporulation, hyphal growth, and fungal biomass were already reduced at the lowest fungicide concentration. A decrease in total sterols, in addition to an increase in the amount of squalene and no accumulation of abnormal sterols, suggests that the sterol pathway is severely slowed down or that squalene epoxidase was inhibited by fenpropimorph in *G. intraradices*. In the root compartment, neither extraradical and intraradical development of the arbuscular mycorrhizal (AM) fungus nor root growth was affected when they were not in direct contact with the fungicide; only hyphal length was significantly affected at 2 mg l^{-1} of fenpropimorph. Our results clearly demonstrate a direct impact of fenpropimorph on the AM fungus by a perturbation of its sterol metabolism.

E. Campagnac · J. Fontaine · A. Lounès-Hadj Sahraoui · F. Laruelle · R. Durand · A. Grandmougin-Ferjani (✉)
Laboratoire de Mycologie Phytopathologie Environnement, Université du Littoral Côte d'Opale,
17 Avenue Blériot, B.P. 699, 62228 Calais Cedex, France
e-mail: grand@univ-littoral.fr

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Introduction

Fenpropimorph belongs to the morpholine group of sterol biosynthesis inhibitors (SBI) and is widely used as a fungicide to control pathogens such as powdery mildew, rusts and leaf blotch diseases of cereals (Leroux 2003). This fungicide also triggers an inhibition of the fungal growth (Debieu et al. 1998, 2000). In the sterol biosynthesis pathway of fungi, fenpropimorph inhibits Δ^8-Δ^7 isomerase and/or Δ^{14} reductase and induces an accumulation of abnormal sterols (Δ^8-sterols and/or $\Delta^{8,14}$-sterols) and a decrease in ergosterol (Baloch et al. 1984; Kerkenaar 1990; Marcireau et al. 1990; Debieu et al. 1992, 2000). However, Marcireau et al. (1990) demonstrated that the fungistatic effect of fenpropimorph on *Saccharomyces cerevisiae* is primarily caused by ergosterol depletion rather than accumulation of Δ^8-sterols or $\Delta^{8,14}$-sterols.

An impact of fenpropimorph on the sterol biosynthetic pathway of nontarget organisms like plants, protozoa, or soil-borne fungi has also been detected. The phytotoxic effects of fenpropimorph application that have been described include growth reduction and necrotic lesions (Costet-Corio and Benveniste 1988; Khalil and Mercer 1991; Grandmougin et al. 1989; He et al. 2003; Schrick et al. 2004). Application of this fungicide to plants inhibits

known target enzymes: cycloeucalenol-obtusifoliol isomerase and Δ^7-reductase. According to the plant species and fungicide concentrations used, Δ^5-sterols can be replaced by biosynthetic intermediates as well as by unusual sterols such as Δ^8-sterols, $\Delta^{8,14}$-sterols, or 9β,19-cyclopropylsterols (Mercer 1993; Rahier and Taton 1997; Hartmann 1998; Hartmann et al. 2002; Benveniste 2004). The side effects of fenpropimorph on several nontarget organisms in soil have also been studied. Soil protozoa were found to be sensitive at recommended field doses (Ekelund et al. 1994; Ekelund 1999; Thirup et al. 2001). In a field study, fenpropimorph significantly reduced sapotrophic fungi (Bjørnlund et al. 2000) and activity of decomposer fungi in soil (Thirup et al. 2001). Furthermore, several studies have observed the effect of fenpropimorph on mycorrhizal plants (Von Alten et al. 1993; Schweiger and Jakobsen 1998; Kjoller and Rosendahl 2000; Schweiger et al. 2001). These studies, using various experimental protocols to evaluate the impact of fenpropimorph, reported an impact on the symbiosis but did not evaluate the effect on the arbuscular mycorrhizal (AM) fungus alone.

Evaluation of the toxicity of pesticides on AM fungi using monoxenic culture systems has been described by several authors (Wan et al. 1998; Zocco et al. 2008; Hillis et al. 2008). Zocco et al. (2008) used this methodology to determine the toxicity of two SBI fungicides (fenpropimorph and fenhexamid). Mycorrhizal roots, cultured in the presence of fenpropimorph, showed a drastic reduction in growth, fungal colonization, and extraradical fungal development. Recently, under the same conditions, we showed that fenpropimorph induces strong modifications of 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) (Campagnac et al. 2008). We concluded that the modifications of mycorrhizal root sterol composition could explain the high toxicity of fenpropimorph to the AM symbiosis. This work did not allow evaluation of the direct impact of fenpropimorph on the AM fungus. Up to now, no studies have evaluated the impact of SBI fungicides on sterol-targeted metabolism of mycorrhizal fungi, especially for AM fungi. Indeed, AM fungi have an unusual sterol composition, and they contain no ergosterol, the predominant sterol of most fungi (Weete 1989), but a mixture of 24-alkylated sterols, mainly 24-methylcholesterol and 24-ethylcholesterol (Grandmougin-Ferjani et al. 1999; Fontaine et al. 2001).

The aim of the present study was to determine whether there is a direct impact of fenpropimorph on sterol biosynthesis pathway of *Glomus intraradices* when the extraradical mycelia alone are in contact with the SBI fungicide. We used monoxenic cultures in order to obtain a large quantity of biological material without risk of contamination by other microorganisms, such as saprophytic or pathogenic fungi, which generally contain ergosterol (Fontaine et al. 2001). Experiments were performed with bi-compartmental plates to separate mycorrhizal roots from extraradical mycelium (St. Arnaud et al. 1996).

Materials and methods

Biological material

Ri T-DNA-transformed chicory roots (*Chicorium intybus* L.) colonized by *G. intraradices* Schenck and Smith (DAOM 197198–MUCL 43194) were grown on modified Strullu–Romand (MSR) medium (Declerck et al. (1998) modified by Strullu and Romand (1986)) solidified with 0.25% (*w*/*v*) gellan gel (Phytagel: Sigma, St Louis, MO, USA) at 27°C in the dark in an inverted position.

SBI fungicide treatments

Fenpropimorph (technical grade) was supplied by BASF AG (Germany). To prepare fungicide-enriched medium, active matter of fenpropimorph was dissolved in acetone (0.5 ml l^{-1} medium) and added to sterilized (121°C for 15 min) MSR medium (80°C) in order to obtain final SBI fungicide concentrations of 0.02, 0.2, 2, or 20 mg l^{-1}. The bottles were agitated by hand and the medium was poured into standard Petri dishes (9 cm). Control treatments, containing MSR medium without SBI fungicide, were supplemented with the same volume of acetone. As the majority of this solvent was evaporated on contact with the MSR medium (Zohrehvand 2005), no toxic effect was observed to the AM fungus (Zocco et al. 2008; Campagnac et al. 2008).

Treatment of *G. intraradices* with fenpropimorph

Fungal growth conditions

Monoxenic cultures were established in bi-compartmental Petri dishes (9 cm) with a watertight plastic wall separating the root compartment (RC) from the fungal compartment (FC) (St. Arnaud et al. 1996). The root compartment was filled with 25 ml MSR medium without fungicide and a piece of mycorrhizal transformed chicory roots was added in the medium. After 3 weeks, the FC was filled with 25 ml MSR medium without fungicide (control) or amended with the different concentrations of fenpropimorph. This compartment was kept root-free (by cutting) and used to investigate the treatment effect on extraradical hypha development.

Effect of fenpropimorph

On mycorrhizal chicory roots without direct contact with the fungicide After 8 weeks in the RC without direct contact with fungicide, the lengths of root and hyphae were measured under a dissecting microscope using the gridline intersect technique, and data were integrated using Tennant's formula (1975). A 1-cm grid was used and each count was converted to total length using the equation: (11/14)×number of intersects×1 (Tennant 1975). The number of spores was determined using the method described by Declerck et al. (2001). Mycorrhizal roots were collected by filtration after solubilization of media (10 min under agitation) in one volume Tris buffer (Tris–HCl 50 mM, pH7.5, EDTA 10 mM) at room temperature. Roots were rinsed with sterile water and frozen at −80°C. One part of the root samples was cleared in KOH (10%) and stained with Chlorazol black E (Brundrett et al. 1994) to quantify arbuscular mycorrhizal colonization using the magnified intersect method (McGonigle et al. 1990).

On extraradical mycelium Eight weeks after the addition of the fungicide in the FC, hyphal length and the number of spores were measured as described above. Medium in the FC was solubilized and the mycelium of the AM fungus was collected by filtration on a 53-μm sieve, rinsed with sterile water, and frozen at −80°C.

Sterol extraction: analyses and identification

Before sterol extraction, mycorrhizal roots and extraradical hyphae of *G. intraradices* were lyophilized for 48 h. The freeze-dried material (20 mg dry weight for roots and 10 mg dry weight for fungal material) was saponified with 3 ml of 6% (*w*/*v*) KOH in methanol at 85°C for 2 h. After the addition of one volume of water, the unsaponifiable fraction was extracted three times with three volumes of hexane and dried under vacuum. Free sterols were submitted to acetylation in a toluene:Ac_2O/pyridine mixture (1:2:1, *v*/*v*/*v*) for 12 h at room temperature. After evaporation of reagents, acetate derivatives were purified on silica gel thin-layer chromatography (TLC) plates ($60F_{254}$; Merck Darstadt, Germany) with dichloromethane as the solvent (one run). Sterol compounds were visualized under UV (254 nm) after spraying with primuline (0.01% *w*/*v*) in aqueous acetone (80%), scraped off, and eluted 30 min with dichloromethane. Final extracts were analyzed using a Perkin Elmer Autosystem gas chromatograph (GC) equipped with a flame-ionization detector (Perkin Elmer, Autosystem, Norwalk, CT, USA) and a EC™-5 (Alltech, Deerfield, USA) capillary column (30 m×0.53 mm i.d.) with hydrogen as carrier gas (3.6 ml min^{-1}). The temperature program included a fast rise from 60°C to 270°C at 20°C min^{-1} and then a rise from 270°C to 300°C at 2°C min^{-1}. Sterol acetates were quantified using an internal standard (not acetylated cholesterol) and identified by gas chromatography–mass spectrometry (GC–MS Varian, Walnutcreek, CA, USA).

Squalene extraction and quantification

Extracted free sterols were separated on TLC plates ($60F_{254}$) with dichloromethane as the solvent (two runs). Squalene was visualized under UV (254 nm), identified with a standard and recovered as above. After evaporation, squalene was purified on TLC plates ($60F_{254}$) with hexane/toluene (19:1; one run) as the solvent and recovered as previously described. Final extracts were analyzed by GC with a temperature program including a fast rise from 60°C to 270°C at 20°C min^{-1} and then a rise from 270°C to 300°C at 5°C min^{-1}. Cholesterol (not acetylated) was used as an internal standard for squalene determination and quantification.

Sterol labeling experiment

Fungal growth conditions

To investigate the ability of the fungus to synthesize sterols in the presence of the SBI fungicide, [1-^{14}C] sodium acetate (65 mCi $mmol^{-1}$, ICN Biomedicals, Inc., USA) was used as a lipid precursor. Monoxenic cultures were established in bi-compartmental Petri dishes (9 cm) as described above. The FC was filled with 25 ml MSR medium without fungicide (control) or amended with different concentrations of fenpropimorph (0.2 and 2 mg l^{-1}). After 8 weeks, ten Petri plates were used for each experimental condition and 10 μCi of [1-^{14}C] sodium acetate was added to each one only in the FC. The incubation period with the labeled lipid precursor was 72 h because during the symbiotic stage the AM fungus exhibited the most active ability to synthesize sterols between 72 and 96 h of incubation (Fontaine et al. 2001). After 72 h of incubation, the medium in the FC was solubilized, and extraradical hyphae were collected and frozen at −80°C as previously described.

Labeled sterol extraction, analysis, and identification

Before sterol extraction, extraradical hyphae of *G. intraradices* were lyophilized for 48 h. Freeze-dried fungal material was extracted by refluxing three times with dichloromethane/methanol (2:1, *v*/*v*) for 3 h. Extracts were combined and dried under reduced pressure. Squalene, free sterols, and their precursors (4,4-dimethyl and 4α-methylsterols) were separated on TLC plates ($60F_{254}$) with

hexane/diethyl ether/glacial acetic acid (78:20:4, *v*/*v*/*v*) as the solvent. Sterols were purified on TLC plates ($60F_{254}$) with dichloromethane as the solvent (two runs). Labeled sterol compounds were detected and quantified by using a radio TLC imaging scanner (AR 2000, Bioscan, WA, USA).

Statistical treatment

ANOVA analysis was carried out with the statistical program STATGRAPHICS release 5.1 (Manugistic, Inc., Rockville, MD, USA). The method used to discriminate between the means was the least significant difference (LSD) test ($P<0.05$). Analysis of variance was conducted on the original data except for the data of colonization and labeling experiments, which were converted to arcsine values before the analysis of variance. Normality of the data was checked with the Levene's test before the use of the multiple comparison procedure.

Heterogeneous variance was detected in the data obtained with the effect of fungicides on sporulation. A nonparametric test such as Kruskal–Wallis for trend was applied. Differences between treatments were estimated by 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 G. Le Pape based on Siegel and Castellan (1988).

Results and discussion

Fenpropimorph drastically reduces the development of *G. intraradices*

Cultures were established in bi-compartmental Petri dishes, one compartment containing mycorrhizal roots (RC) on MSR medium, and the other compartment containing only fungus (FC) on MSR medium amended with fenpropimorph (0.02, 0.2, 2, or 20 mg l^{-1}). Dry weight of *G. intraradices* grown 8 weeks in the FC is presented in Table 1. Biomass was drastically reduced in the presence of increasing concentrations of fenpropimorph. Dry weight at the lowest concentrations of fenpropimorph is reduced three times as compared to the control and is close to zero at 20 mg l^{-1} of fenpropimorph. The hyphal length was also significantly reduced in the presence of fenpropimorph (Table 1). The hyphal length obtained with 0.02 mg l^{-1} of fenpropimorph (349 cm) is almost half of that in the control treatment (661 cm), and at the highest concentration of fenpropimorph, hyphal length of *G. intraradices* is very low. A decrease in spore production was observed (Table 1): at the highest concentration of fenpropimorph, spore production was close to 70 spores, whereas for the control treatment, spore production was near 4,000. Fenpropimorph not only impacted on spore production but also perturbed the architecture of the mycelium under the same culture conditions (Zocco et al. 2008). Studies done on fenpropimorph have shown a drastic decrease in the growth of pathogenic fungi, demonstrating the high efficiency of this compound as a fungicide (Baloch et al. 1984; Debieu et al. 1998, 2000).

Table 1 Extraradical development of *G. intraradices* in the absence or presence of fenpropimorph after 8 weeks of growth: biomass, hyphal length, and spore production

Fenpropimorph (mg l^{-1})	Dry weight (mg)	Hyphal length (cm)	Spore number
Control	15.1±1.7a	661.1±24.0a	3856.2±158.2a
0.02	5.7±1.1b	349.5±55.9b	1831.4±507.3b
0.2	4.3±0.7bc	237.4±38.2c	843.0±330.2c
2	1.4±0.2c	149.9±20.1c	143.4±63.7c
20	0.5±0.1c	140.2±42.7c	68.6±30.9c

Values are means ± standard error of three different experiments. Different letters within columns indicate significant differences ($P<0.05$) according to the LSD test for the dry weight and hyphal length data and Siegel–Castellan test of multiple comparisons for sporulation data

Sterol modifications in *G. intraradices* grown in the fungal compartment treated with fenpropimorph

Sterol composition of *G. intraradices* grown in the presence of different concentrations of fenpropimorph (0.02, 0.2, or 2 mg l^{-1}) during 8 weeks is shown in Table 2. In the control treatment (without fenpropimorph), major sterols of *G. intraradices* were identified as a mixture of 24-alkylsterols: 24-methylcholesterol (460.7 μg g^{-1}), 24-ethylcholesterol (155.8 μg g^{-1}), and 24-ethylcholesta-5,22-dienol (3.9 μg g^{-1}). No ergosterol was detected. This result is in agreement with previous studies (Grandmougin-Ferjani et al. 1999, 2005; Fontaine et al. 2001, 2004). At the higher fenpropimorph concentration and compared to the control, significant decreases in 24-methylcholesterol (85%) and in 24-ethylcholesterol (75%) were observed. For 20 mg l^{-1} of fenpropimorph, the limited amount of fungal material was insufficient to perform the sterol analysis.

This study showed a decrease of total sterol amounts in *G. intraradices*, but no apparition of abnormal sterols was described for most fungi. In fact, fenpropimorph was shown to inhibit sterol target enzymes (the Δ^8-Δ^7 isomerase and the Δ^{14} reductase) in pathogen fungi, inducing an accumulation of abnormal sterols: Δ^8-sterols and $\Delta^{8,14}$-sterols and a decrease in ergosterol (Baloch et al. 1984; Baloch and Mercer 1987; Debieu et al. 1998, 2000). Only Engels and

Table 2 Sterol composition of *Glomus intraradices* grown in the fungal compartment in the absence or presence of fenpropimorph (a) and of mycorrhizal roots colonized by *G. intraradices* grown without the SBI fungicide (b)

	Fenpropimorph (mg l^{-1})							
	Control (μg g^{-1})	Percentage	0.02 (μg g^{-1})	Percentage	0.2 (μg g^{-1})	Percentage	2 (μg g^{-1})	Percentage
a. Glomus intraradices								
4-Demethylsterols								
Cholesterol	8±1.8a	1.3a	Tr	Tr	5.1±1.9a	3.3a	–	–
24-Methylcholesterol	460.7±48a	72.4a	201.7±35.9b	70.0a	105.2±43.4bc	67.4a	73.7±5.9c	56.2a
24-Ethylcholesta-5,22-dienol	3.9±0.2a	0.6a	4.5±1.5a	1.6a	1.5±0.9a	0.9a	–	–
24-Ethylcholesterol	155.8±17.4a	24.5a	81.5±12.5b	28.3a	44.3±15.8b	28.4a	38±6.4b	29.0a
Sterol nonidentified	7.6±4.7a	1.2a	–	–	–	–	19.5±13.4a	14.8a
Total sterols (μg g^{-1} dry weight)	636±72a		288±51b		156±62b		131±26b	
b. Mycorrhizal roots								
4-Demethylsterols								
Cholesterol	5.2±1.5a	0.6a	7±2a	1.1a	10±2.2a	0.9a	4±4.6a	0.4a
24-Methylcholesterol[a]	200.1±78.1a	24.5a	171±37.3a	25.5a	254.8±20.7a	24a	199.1±71a	22.1a
Stigmasterol	401.1±127.1a	49.2a	343.4±82.7a	51.2a	544.7±85.8a	51.2a	462.9±175.4a	51.5a
24-Epiclerosterol	13.9±5.7a	1.7a	10.2±2.7a	1.5a	9.3±8.6a	0.9a	21.3±19.8a	2.4a
Sitosterol	195.6±68.4a	24a	138.8±28.7a	20.7a	245±77.3a	23a	211.9±84.3a	23.6a
Total sterols (μg g^{-1} dry weight)	816±162a		670±77a		1,064±112a		899±177a	

Data are presented in part (a) as means±standard error (n=4) and in (b) as means±standard deviation (n=4). Different letters within columns indicate significant differences (P<0.05) according to the LSD test

Tr traces, amounts <0.5% or 1 μg g^{-1}, – not detected

[a] Mixture of 24-methylcholesterol, 24-methylecholesterol, and 24-methyldesmosterol not separated in our GC conditions

De Waard (1998) showed the absence of a specific sterol profile when *Blumeria graminis* was treated with fenpropimorph: in different isolates after a curative treatment with fenpropimorph, no accumulation of $\Delta^{8,14}$-sterols or Δ^{8}-sterols was observed and sterol content was found to be halved. Interestingly, this obligate biotrophic fungus did not synthesize ergosterol but a mixture of 24-ethylsterols (24-ethylcholesta-5,22-dienol, 24-ethylcholesterol, and Δ5-avenasterol) in young conidia and 24-methylsterols (24-methylenecholesterol and episterol) in old conidia (Senior et al. 1995; Engels and De Waard 1998; Muchembled et al. 2000).

Our results clearly show a fenpropimorph dose-dependent decrease in the sterol content of the AM fungus. The sterol content in the control treatment was 636 μg g^{-1}, and this decreased by 55%, 75%, and 80% at 0.02, 0.2, and 2 mg l^{-1} of fenpropimorph, respectively.

The amount of squalene in *G. intraradices* grown in the presence of 0, 0.2, and 2 mg l^{-1} fenpropimorph is shown in Table 3. In the control treatment, squalene content was about 40 μg g^{-1}. After fenpropimorph application, a significant increase of 8.5-fold was observed at 2 mg l^{-1} fenpropimorph. Interestingly, the ratio of total sterol/squalene indicates 15.9-fold more sterol than squalene in the control treatment (Table 3). This ratio decreases to 2.7-fold in the presence of 0.2 mg l^{-1} fenpropimorph, and inversely 2.6-fold more squalene than sterol was found in the fungus treated with 2 mg l^{-1}. The fungicide induces an accumulation of squalene in *G. intraradices* corresponding approximatively to a decrease of 40-fold in the ratio total sterol/squalene. These effects of fenpropimorph application on the total amount of sterol and on squalene, the precursor of the first step of cyclization in sterol biosynthesis, suggest a less active sterol metabolism.

Ability of *G. intraradices* to synthesize sterols

Figure 1 shows the ability of *G. intraradices* to synthesize sterols in the absence and presence of different concentrations of fenpropimorph (0.2 and 2 mg l^{-1}) using [1-^{14}C] sodium acetate labeling as a lipid precursor. The radiolabeled acetate was incorporated into 4-demethylsterols, which are the typical end-products of the sterol biosynthetic pathway, and into their precursors squalene, 4,4-dimethyl- and 4α-methylsterols. A decrease of 70% in the amount of radioactivity incorporated into 4-demethylsterols was observed for the fungus treated with fenpropimorph. The decline in the capacity of *G. intraradices* to synthesize sterols did not result from a decrease in the uptake of labeled substrate by the fungus from the medium amended with fenpropimorph. For all treatments, about 30% of the radiolabeled acetate initially added to the MSR medium was taken up by the extraradical mycelium (data not shown). These unusual results obtained for *G. intraradices* treated with fenpropimorph, particularly the important decrease in the amount of total sterol, demonstrate an inhibition of the sterol biosynthetic pathway which was confirmed by the low radioactivity associated with 4-demethylsterols using [1-^{14}C] sodium acetate labeling as a lipid precursor.

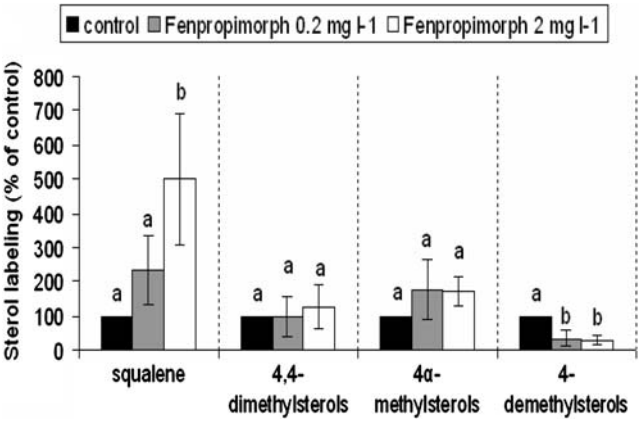

Fig. 1 Effect of fenpropimorph on the incorporation of radioactivity from [1-^{14}C] sodium acetate into squalene, sterols, and their precursors in *G. intraradices* after an incubation of 72 h. Sterol labeling was expressed as percentage of control values. Results are obtained from three different experiments; *error bars* represent ± 1 SD. *Different letters* within columns indicate significant differences ($P<0.05$) according to the LSD test

Table 3 Squalene amounts and the total sterol/squalene ratio in *G. intraradices* grown in the fungal compartment in the absence or presence of fenpropimorph

	Fenpropimorph (mg l^{-1})		
	Control	0.2	2
Squalene (μg g^{-1} dry weight)	40±7a	57±10a	338±73b
Ratio total sterol/squalene	15.9	2.74	0.39

Data are presented as means ± standard error ($n=3$). Different letters within columns indicate significant differences ($P<0.05$) according to the LSD test

In the fungicide-treated fungus, radioactivity recovered in squalene was significantly higher than in the control. At 2 mg l^{-1} of fenpropimorph, squalene labeling was 5-fold more important than in the control. This result corroborates the squalene accumulation described above. We can propose several hypotheses to explain the squalene accumulation in *G. intraradices* cultured in the presence of fenpropimorph. The first is an inhibition of squalene epoxidase by fenpropimorph. This enzyme catalyzes the first oxygenation step in sterol biosynthesis and converts squalene into 2,3-oxidosqualene which is then cyclized by a subsequent enzyme to form lanosterol in fungi. Hartmann (1998) noted that in plants, a net decrease in the amount of

free sterols could be due to an inhibition at the early steps of the biosynthesis pathway, such as the squalene epoxidase. The hypothesis concerning this enzyme as a potential target of fenpropimorph was also suggested for *Nectria haematococca* var *cucurbitae* and *Fusarium* (Ziogas et al. 1991; Debieu et al. 1992, 1998). Normally, squalene epoxidase is the target of another SBI: allylamines. Its inhibitory activity is accompanied by an accumulation in squalene and a lack of ergosterol in fungi or yeasts (Ryder 1987, 1991; Ruckenstuhl et al. 2007). However, it is also hypothetically possible that an accumulation of squalene is not directly related to an inhibition of squalene epoxidase, but is a side effect resulting from retro-inhibition of biosynthesis by sterol intermediates. In this latter hypothesis, squalene accumulation could be more of a consequence of AM fungal growth inhibition rather than an inhibition of the sterol biosynthetic pathway.

Relations between observed sterol modifications and fungal growth inhibition

The drastic reduction in *G. intraradices* development observed in the presence of fenpropimorph could originate from the decrease in total sterol levels, as described by Marcireau et al. (1990) who attributed the fungistatic effect of fenpropimorph to the depletion of ergosterol rather than the accumulation of abnormal sterols. However, the decrease in AM fungal growth could also be explained by toxicity of squalene accumulation, as suggested for *Candida* ssp. (Ryder and Favre 1997). The resulting ergosterol depletion and squalene accumulation are known in fungi to affect membrane structure and function (Georgopapadakou and Walsh 1996), and several authors have discussed the possibility of squalene accumulation at toxic concentrations in fungal hyphae resulting in cell death (Franklin and Snow 2005). Nevertheless, squalene is a neutral lipid which can pass through membranes easily and it can be stocked in high amounts in intracellular lipid droplets, generally without any deleterious effect, in plant cells treated with terbinafine (Wentzinger et al. 2002). In the present case, the moderate squalene accumulation observed in *G. intraradices* seems to be more of a consequence of AM fungal growth inhibition rather than due to its potential toxicity.

No indirect effect of fenpropimorph on mycorrhizal roots and fungal development grown in root compartment

Many studies conducted on the dissipation of fenpropimorph have demonstrated a volatilization of fenpropimorph of nearly 6% and 7% of the fungicide within 24 h from the soil (Rüdel 1997) and from plant surfaces (Leistra et al. 2006). For this reason, the impact of fenpropimorph on the RC was also investigated in order to check the eventual indirect impact of fenpropimorph on mycorrhizal roots.

The development of mycorrhizal roots in the RC is presented in Table 4. Biomass and root length of mycorrhizal roots were not affected when they were not in direct contact with the fungicide. Concerning fungal development, no significant differences in sporulation and in root colonization were observed, whereas hyphal length in the RC was significantly affected by 2 mg l^{-1} of fenpropimorph when compared to the control treatment. Zocco et al. (2008) and Campagnac et al. (2008) showed that only the growth of roots incubated directly in contact with fenpropimorph is strongly inhibited. In this case, biomass and root colonization decreased strongly, and morphology is also modified. This suggests that the impact of fenpropimorph on roots exists only when the fungicide is in direct contact. Once fenpropimorph is incorporated into the MSR medium, there is no diffusion into the air and no volatile impact. Nevertheless, we observed that at 2 mg l^{-1} of fenpropimorph in the FC, hyphal length in the RC decreased. Extraradical mycelium was the only link between both compartments. This could suggest that fenpropimorph, at high concentrations, may be taken up and carried via mycelium from the FC to the RC, so inhibiting extraradical mycelium development without disrupting root colonization.

Table 4 Indirect effect of fenpropimorph in the root compartment after 8 weeks on biomass and length of roots and on fungal development: mycorrhizal colonization, hyphal length, and spore production

Root			Glomus intraradices		
Fenpropimorph (mg l^{-1})	Dry weight (mg)	Root length (cm)	Mycorrhizal colonization (%)	Hyphal length (cm)	Spore number
Control	57.0±5.2a	141.4±7.3a	42.2a	598.1±37.7a	1,739.2±280.8a
0.02	57.9±4.5a	156.8±15.5a	32.8a	672.4±61.6a	1,814.6±459.7a
0.2	55.6±3.9a	156.4±6.0a	29.2a	603.6±71.2a	1,935.2±304.8a
2	54.5±4.0a	149.3±12.2a	34.8a	331.1±56.0b	1,812.8±224.2a
20	55.9±5.5a	130.9±20.9a	29.7a	270.9±39.1b	1,282.2±286.9a

Data are presented as means ± standard error (n=4). Different letters within columns indicate significant differences (P<0.05) according to the LSD test

No indirect effect of fenpropimorph on the sterol profile of mycorrhizal roots

The sterol profile of mycorrhizal roots growing without direct contact with the fungicide is presented in Table 2. In the control treatment, the typical Δ^5-sterols of roots were identified: stigmasterol, sitosterol, and 24-methylcholesterol. Traces of 24-methylene cholesterol were also detected. This is very probably due to the presence of the intraradical AM fungus. This result is in agreement with those obtained by Fontaine et al. (2004). With fungicide treatment in the FC, no changes in sterol composition of colonized roots were observed. Three major sterol compounds sitosterol, 24-methylcholesterol, and stigmasterol were found in the same proportions in all treatments, and no abnormal sterols were observed. Campagnac et al. (2008) have shown that the sterol profile of carrot roots was completely modified when they were in contact with fenpropimorph. The accumulation of the abnormal sterols, 9β,19-cyclopropylsterols $\Delta^{8,14}$-sterols, Δ^8-sterols, and Δ^7-sterols was observed and four target enzymes, cycloeucalenol-obtusifoliol isomerase, Δ^{14} reductase, Δ^8-Δ^7 isomerase, and Δ^7 sterol C 5(6) desaturase, were inhibited. Finally, as for root development, the sterol profile of mycorrhizal roots was not affected without direct contact with fenpropimorph. This result excludes transport of fenpropimorph to the intraradical fungus and its action at a distance.

No translocation of sterols between intraradical and extraradical hyphae

In 2002, Bago et al. have shown a significant bi-directional translocation in hyphae of lipid bodies containing storage lipids. They concluded that the large amounts of lipids are translocated between the intraradical and the extraradical mycelia with a possible recirculation of these lipid bodies throughout the fungus. These structures delimited by a phospholipid monolayer contain predominantly triacylglycerol and steryl esters which correspond to the storage form of sterols (Zweytick et al. 2000). In our study, the sterol pathway slowdown observed in the extraradical mycelium cultivated in the presence of fenpropimorph and the negative SBI impact on extraradical mycelium development suggest an absence of transport of sterol compounds between the intraradical and extraradical hyphae. One might expect that inhibition of the sterol biosynthesis could be offset by the sterol biosynthesis in intraradical mycelia but it is not the case.

In conclusion, fenpropimorph clearly affects the development and sterol metabolism of *G. intraradices*, a nontarget organism. In a previous work, we showed that the modification of phytosterol composition could explain the high toxicity of fenpropimorph to the AM symbiosis (Campagnac et al. 2008). In this work, we provide the first evidence of a direct impact of fenpropimorph on the AM fungus by disturbing its sterol metabolism.

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