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Differential effects of fenpropimorph and fenhexamid, two sterol biosynthesis inhibitor fungicides, on arbuscular mycorrhizal development and sterol metabolism in carrot roots

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ABSTRACT

Sterols composition of transformed carrot roots incubated in presence of increasing concentrations of fenpropimorph (0.02; 0.2; 2 mg l^{-1}) and fenhexamid (0.02; 0.2; 2; 20 mg l^{-1}), colonized or not by *Glomus intraradices* was determined. In mycorrhizal roots treated with fenpropimorph, normal Δ^5-sterols were replaced by 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). After application of fenpropimorph, a drastic reduction of the mycorrhizal root growth, root colonization and extraradical fungal development was observed. Application of fenhexamid did not modify sterol profiles and the total colonization of roots. But the arbuscule frequency of the fungal partner was significantly affected.

Comparison of the effects caused by the tested fungicides indicates that the usual phytosterols may be involved in symbiosis development. Indeed, observed modifications of root sterols composition could explain the high fenpropimorph toxicity to the AM symbiosis. However, the absence of sterolic modifications in the roots treated with fenhexamid could account for its more limited impact on mycorrhization.

1. Introduction

Fungicides are important for effective control of plant diseases which cause yield reductions of crops. Most of fungicides act directly on essential fungal functions such as respiration, lipid synthesis or cell division (Leroux, 2003). Consequently they can exhibit undesirable effects on non-target organisms. Among fungicides, sterol biosynthesis inhibitor (SBI) fungicides are the leading group used in agriculture (Hewitt, 1998). Four main classes can be distinguished according to their action target site: (i) squalene epoxidation (e.g. naftifine, terbinafine, tolnaftate), (ii) Δ^{14} demethylation or DMIs (e.g. imazalil, prochloraz, triadimenol, propiconazole), (iii) Δ^{14}-reduction and/or $\Delta^8 \rightarrow \Delta^7$ isomerisation (e.g. fenpropidine, fenpropimorph, tridemorph), (iiii) C4 demethylation (e.g. fenhexamid) (Leroux, 2003). In the present work, two SBI belonging to the last two groups of fungicides were used: systemic morpholine fungicide, fenpropimorph, has been greatly used for the control of pathogen fungi in crops and a recent fungicide, fenhexamid, which is highly effective against *Botrytis* and related fungal pathogens (Rosslenbroich, 1999). Most of plant species including agricultural and horticultural crops establish symbiosis with arbuscular mycorrhizal (AM) fungi (Brundrett, 2002). This association is beneficial to plant growth and resistance against abiotic or biotic stresses (Smith and Read, 1997). However, agricultural practices do not allow the use or the survival of AM fungi in the soil. In fact, the use of fungicides is generalized in modern agriculture for the control of fungal diseases but these chemicals can exhibit undesirable effects on non-target plant-beneficial micro-organisms. For this reason, it is mandatory to study the impact of fungicides on the functioning of the symbiosis in order to preserve beneficial effects of mycorrhizae to plant nutrition while keeping inhibition of phytopathogen fungi.

Many studies on the effects of SBI fungicides on mycorrhizal plants have been conducted and gave contradictory results on the plant growth, on AM fungal development (root colonization, sporulation, germination), or on the functioning of symbiosis (Dodd and Jeffries, 1989; Von Alten et al., 1993; Schweiger and Jakobsen, 1998; Kjoller and Rosendahl, 2000; Schweiger et al., 2001).

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The diversity in experimental procedure of these studies (plant species, environmental growth conditions, fungicide formulation, application methods, micro-organisms present in soil...) led to some difficulties to compare obtained results, and to conclude on the impact of SBI fungicides on AM fungi (Sancholle et al., 2001). Monoxenic cultivation system as an easy and a reproducible technique to evaluate toxicity of pesticides on AM fungi, has been demonstrated by several authors (Wan et al., 1998; Zocco et al., 2008). Recently Zocco et al. (2008), showed the inhibitory effects of two SBI fungicides, i.e. fenpropimorph and fenhexamid on fungal and root development.

But so far no study has shown the impact of these fungicides on sterol's metabolism. Only the use of monoxenic cultures permits investigation of sterols content without risk of contamination (Fontaine et al., 2004). Indeed, monoxenic cultures allow obtaining a large quantity of biological material, free of contaminant micro-organisms as these saprophytic or phytopathogenic fungi contain generally ergosterol.

In the present work, we used the monoxenic cultivation system in order to link up the direct impact of fenpropimorph and fenhexamid, on the symbiosis development (carrot roots/*Glomus intraradices*) and their impact on the sterol target metabolism of colonized roots.

2. Results and discussion

2.1. Effect of SBI fungicides on root sterol profile

Sterols composition of transformed carrot roots incubated in presence of increasing concentrations of fenpropimorph (0.02; 0.2; 2 mg l^{-1}) and fenhexamid (0.02; 0.2; 2; 20 mg l^{-1}), colonized or not by *G. intraradices* was determined (Table 1). In the absence of fungicide, sterols of non-mycorrhizal roots were identified as stigmasterol, sitosterol, 24-methylcholesterol, isofucosterol and 24-epiclerosterol, the typical Δ^5-sterols in carrot roots. Traces of some precursors of the sterol biosynthetic pathway were also detected, i.e., cycloartenol, 24-methylene cycloartanol. After colonization of roots by *G. intraradices*, the same mixture of Δ^5-sterols was detected. These results are in agreement with those reported by Fontaine et al. (2001). As shown before, *G. intraradices* contains a mixture of 24-alkylsterols and no ergosterol (Grandmougin-Ferjani et al., 1999; Fontaine et al., 2001; Olsson et al., 2003). No major change in sterol composition of colonized roots was highlighted in our experiment. Traces of 24-methylene cholesterol, probably originating from the fungus (Fontaine et al., 2001) were detected in colonized roots (Table 1b). This compound was not separated from 24-methylcholesterol under our GC analysis conditions. It was previously established that the 24-methyl/methylene sterols are an appropriate indicator of colonization by AM fungi of transformed roots (Fontaine et al., 2004), nevertheless no increase of the relative percentage of 24-methyl/methylene cholesterol was observed in colonized root This low content of this indicator was probably due to the moderate colonization level by *G. intraradices* of the roots in our experiment (Fig. 4).

With fenpropimorph, a decrease of normally occurring Δ^5-sterols was observed with increasing concentrations of fungicide in non-mycorrhizal roots (Table 1a). At 2 mg l^{-1} of fenpropimorph, Δ^5-sterols represented only 46% (0.53 mg g^{-1} dry wt) of total sterol against 100% (1.74 mg g^{-1} dry wt) in non-colonized roots. A similar decrease of Δ^5-sterols was observed with mycorrhizal roots (Table 1b). Δ^5-Sterols represented 38% of total sterols against 100% in mycorrhizal roots. Normal sterols were replaced by 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).

In mycorrhizal roots, the same modifications in the sterol profile were highlighted indicating the same sensitivity to the fenpropimorph of the mycorrhizal roots. No significant difference was shown in mycorrhizal roots treated by 2 mg l^{-1} of fenpropimorph compared to the non-colonized roots between the relative proportions of detected Δ^5-sterols and abnormal sterols except sitosterol which was significantly reduced (from 31% to 14% of total sterols, respectively).

In contrast, with fenhexamid, no accumulation of abnormal sterols was observed in colonized and non-colonized roots and relative proportions of sterols carrot roots remained unchanged with treatments. In 2001, Debieu et al. have shown that *Botryotinia fuckeliana* was grown in presence of fenhexamid, ergosterol content was reduced and three 3-keto compounds (4α-methylfecosterone, fecosterone and episterone) were accumulated. The authors concluded that sterone accumulation was due to inhibition of a 3-ketoreductase, involved in C4 sterol demethylation. In our experiment, no sterones were detected. Only a significant decrease in the total amount of sterols was observed with increasing concentrations of fenhexamid in the non-colonized and colonized roots.

2.2. Effect of SBI fungicides on the root growth

When carrot roots were incubated with fenpropimorph during 8 weeks a deep modification of root morphology was observed such as a yellowing of roots and an inhibition of secondary roots. These observations were in accordance with those reported by Zocco et al. (2008). Significant reduction of root biomass was observed (Fig. 1a). Whatever fenpropimorph concentration in the medium, dry weight of non-mycorrhizal or mycorrhizal roots by *G. intraradices* was significantly lower as compared to controls without fungicide. Fig. 2a shows the effect of different concentrations of fenpropimorph on carrot roots length, colonized or not. Presence of fenpropimorph in the culture medium caused a very important reduction of root length and biomass up to 97% and 100%, respectively, at highest concentration. This reduction of growth was shown both in colonized and non-colonized roots. With fenhexamid, no significant reduction in the root development between the colonized or non-colonized roots incubated with the fungicide was observed (Figs. 1b and 2b). In 2008, Zocco et al. showed on the same material an inhibition in the root fresh weight after 4 weeks of culture in presence of fenhexamid. The reduction of growth was reversible and demonstrated a phytostatic effect on roots exposed to fenhexamid. This seems to suggest that fenhexamid caused only a delay in the root growth and not a definitive inhibition. On the other hand, an influence of fenhexamid on hydric nutrition of roots cannot be excluded. This is suggested by a decrease of the fresh weight and not of the dry weight of the roots in contact with the fungicide.

2.3. Effect of SBI fungicides on fungal development

The percentage of mycorrhization in control treatment (i.e. without fungicide) reached 32% (Fig. 3a). While at all fenhexamid concentrations, no significant differences were observed in the proportion of colonization, root colonization was reduced by the fenpropimorph treatment. Only 7% of colonization was obtained with the lowest concentration of fenpropimorph used 0.02 mg l^{-1}. Colonization level reached nearly zero level from 0.2 mg l^{-1}. A significant decrease of arbuscules amount inside roots was observed from 0.2 mg l^{-1} of fenhexamid and from 2 mg l^{-1} of fenpropimorph (Fig. 3b). SBI fungicides seem to affect the symbiosis establishment and/or functioning. Indeed, arbuscules are short-lived structures believed to have a turnover rate of one to two weeks. Furthermore, these fungal structures are found preferen-

Table 1
Sterol composition of carrot roots colonized or not by *G. intraradices* grown in presence of SBI fungicides

Sterols	Control		Fenpropimorph (mg l^{-1})						Fenhexamid (mg l^{-1})							
			0.02		0.2		2		0.02		0.2		2		20	
	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%
(a) Non-mycorrhizal roots																
Δ^5-Sterols																
24-Methylcholesterol	221 ± 46[a]	13	100 ± 9[b]	6	35 ± 7[c]	3	40 ± 35[c]	3	205 ± 151[a]	15	139 ± 63[a]	12	147 ± 53[a]	13	114 ± 47[a]	12
Stigmasterol	929 ± 165[a]	54	411 ± 30[b]	26	179 ± 28[bc]	15	140 ± 28[c]	12	724 ± 431[ab]	53	651 ± 318[ab]	54	648 ± 172[ab]	56	544 ± 270[b]	57
24-Epiclerosterol	17 ± 9	1	–	–	–	–	–	–		–		–	–	–	–	–
Sitosterol	510 ± 97[a]	29	443 ± 51[ab]	28	318 ± 46[b]	26	354 ± 33[b]	31	376 ± 121[ab]	27	375 ± 120[ab]	31	330 ± 79[b]	28	270 ± 106[b]	29
Isofucosterol	58 ± 21	3	68 ± 18	4	–	–		–	26 ±20	2	23 ±27	2	27 ± 4	2	15 ± 18	2
Precursors																
Cycloartenol	tr[*]	tr	tr	tr	tr	tr	tr	tr		–		–		–		–
24-Methylene cycloartanol	tr	tr	tr	tr	tr	tr	tr	tr		–		–		–		–
Abnormal sterols																
Ergosta-8,14-dienol	–[**]	–	38 ± 14	2	56 ± 61	4	36 ± 2	3	–	–	–	–	–	–	–	–
Stigmasta-7,22-dienol	–	–	168 ± 47	10	214 ± 20	17	87 ± 15	7	–	–	–	–	–	–	–	–
24-Methylpollinastanol	–	–	158 ± 36	10	43 ± 7	4	113 ± 16	10	–	–	–	–	–	–	–	–
Stigmasta-8,14-dienol	–	–	-	–	114 ± 3	9	226 ± 10	19	–	–	–	–	–	–	–	–
Δ^8 Sitosterol	–	–	189 ± 51	12	254 ± 7	21	112 ± 8	10	–	–	–	–	–	–	–	–
Non-identified	–	–	30 ± 7	2	6 ± 9	1	55 ± 47	5	43 ± 35	3	11 ± 5	1	9 ± 2	1	–	–
Total sterols (mg g^{-1} dry wt)	1.74[a]		1.60[a]		1.22[b]		1.16[b]		1.37[ab]		1.21[ab]		1.16[b]		0.95[b]	
±s.d.	0.14		0.13		0.11		0.06		0.64		0.51		0.27		0.42	
Total Δ^5-sterols (mg g^{-1} dry wt)	1.74[a]		1.02[b]		0.79[bc]		0.53[c]		1.33[ab]		1.19[b]		1.15[b]		0.94[b]	
±s.d.	0.14		0.03		0.37		0.07		0.56		0.44		0.22		0.37	
(b) Mycorrhizal roots																
Δ^5-Sterols																
24-Methylcholesterol [***]	233± 48[a]	15	162 ± 39[b]	11	65 ± 1[b]	7	31 ± 17[b]	4	172 ± 89[ab]	18	127 ± 109[ab]	21	130 ± 75[b]	16	114 ± 62[b]	15
Stigmasterol	828 ± 98[a]	52	641 ± 112[b]	46	307 ± 10[c]	32	172 ± 68[c]	20	641 ± 311[ab]	52	518 ± 440[ab]	50	494 ± 285a[b]	60	431 ± 222[b]	59
24-Epiclerosterol	29 ± 12	2	26 ± 45	2	–	–	–	–		–		–	–	–	–	–
Sitosterol	447 ± 41[a]	28	355 ± 97[a]	25	218 ± 93[b]	23	115 ± 28[b]	14	255 ± 91[b]	28	248 ± 241[b]	24	187 ± 103[b]	23	165 ± 119[b]	23
Isofucosterol	42 ± 20	3	tr	tr	–	–		–	–	–	–	–	tr	tr	9 ± 16	1
Precursors																
Cycloartenol	tr[*]	tr	tr	tr		tr		tr	–	–	–	–	–	–	–	–
24-Methylene cycloartanol	tr	tr	tr	tr		tr		tr	–	–	–	–	–	–	–	–
Abnormal sterols																
Ergosta-8,14-dienol	–[**]	–	–	–	11 ± 16	1	71 ± 66	8	–	–	–	–	–	–	–	–
Stigmasta-7,22-dienol	–	–	tr	tr	140 ± 65	15	96 ± 35	11	–	–	–	–	–	–	–	–
24-Methylpollinastanol	–	–	95 ± 34	7	44 ± 62	5	104 ± 73	12	–	–	–	–	–	–	–	–
Stigmasta-8,14-dienol	–	–	–	–	14 ± 19	tr	122 ± 107	15	–	–	–	–	–	–	–	–
Δ^8 Sitosterol	–	–	124 ± 21	9	165 ± 108	17	116 ± 40	14	–	–	–	–	–	–	–	–
	–														–	
Non-identified	–	–	–	–	–	–	18 ± 31	2	31 ± 3	2	53 ± 33	5	11 ± 11	1	11 ± 7	2
Total sterols (mg g^{-1} dry wt)	1.58[a]		1.40[ab]		0.96[bc]		0.84[c]		1.10[ab]		0.95[ab]		0.82[b]		0.73[b]	
± s.d.	0.17		0.17		0.18		0.40		0.49		0.78		0.46		0.38	
Total Δ^5-sterols (mg g^{-1} dry wt)	1.58[a]		1.18[b]		0.59[c]		0.32[c]		1.07[ab]		0.89[b]		0.81[b]		0.72[b]	
± s.d.	0.17		0.21		0.10		0.11		0.49		0.79		0.46		0.37	

Sterols sharing the same letters (a, or b, or c) are not significantly different according to the LSD test ($P < 0.05$) ($n = 4$).
[*] tr, traces, amounts <0.5% or 1 µg g^{-1}.
[**] –, not detected.
[***] 24-methylcholesterol, mixture of 24-methyl/methylene cholesterol.

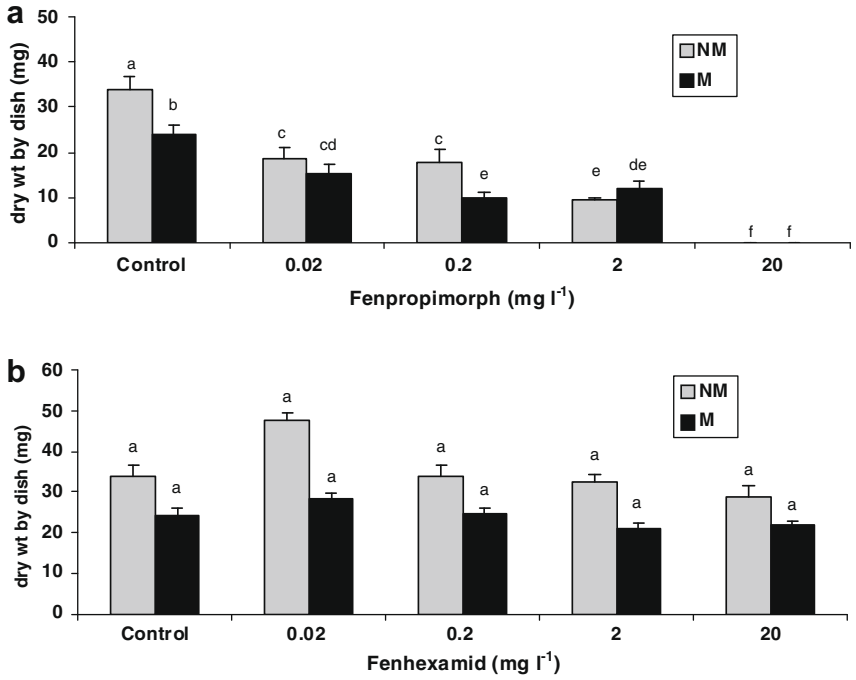

Fig. 1. Biomass of transformed carrot roots (*Daucus carota* L.) colonized or not by *G. intraradices* (mycorrhizal roots M or non-mycorrhizal roots NM) after 8 weeks of growth in the absence or presence of fenpropimorph (a) or fenhexamid (b). The means were obtained from four replicates. In all plots, data are presented as mean ± s.e. Bars sharing the same letters are not significantly different according to the LSD test ($P < 0.05$).

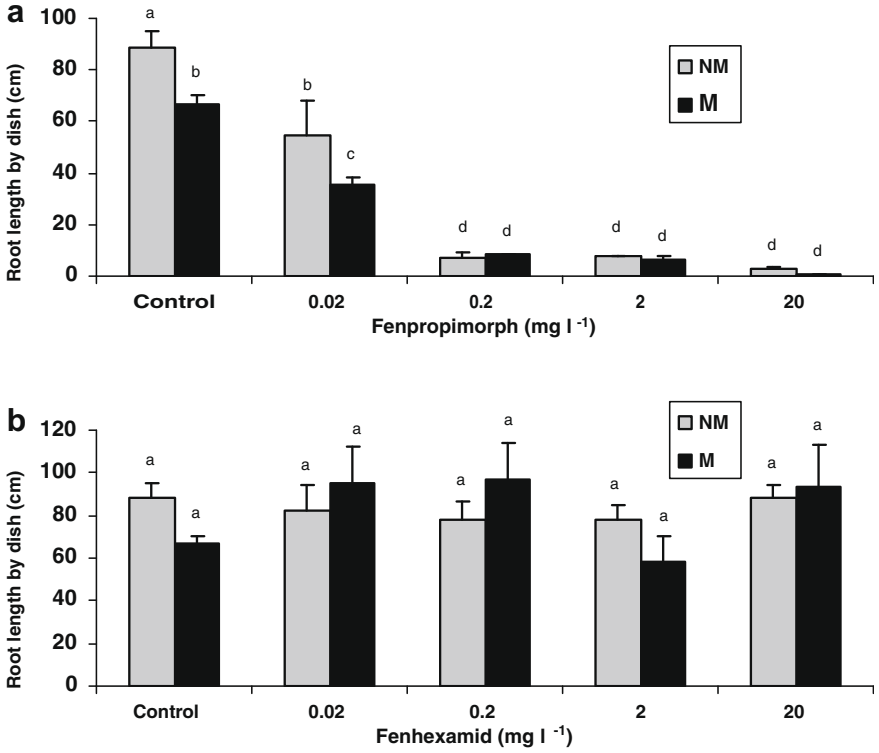

Fig. 2. Root length of transformed carrot roots (*Daucus carota* L.) colonized or not by *G. intraradices* (mycorrhizal roots M or non-mycorhizal roots NM) after 8 weeks of growth in the absence or presence of fenpropimorph (a) or fenhexamid (b). The means were obtained from four replicates. In all plots, data are presented as mean ± s.e. Bars sharing the same letters are not significantly different according to the LSD test ($P < 0.05$).

tially in young, thin roots during early stages of root colonization. This could explain their low percentage in the control (Smith and Read, 1997; Van Aarle and Olsson, 2003; De Souza et al., 2005).

The number of spores produced by colonized roots growing in synthetic media supplemented or not with two fungicides after 8 weeks of culture is presented in Fig. 4a. With fenpropimorph, a

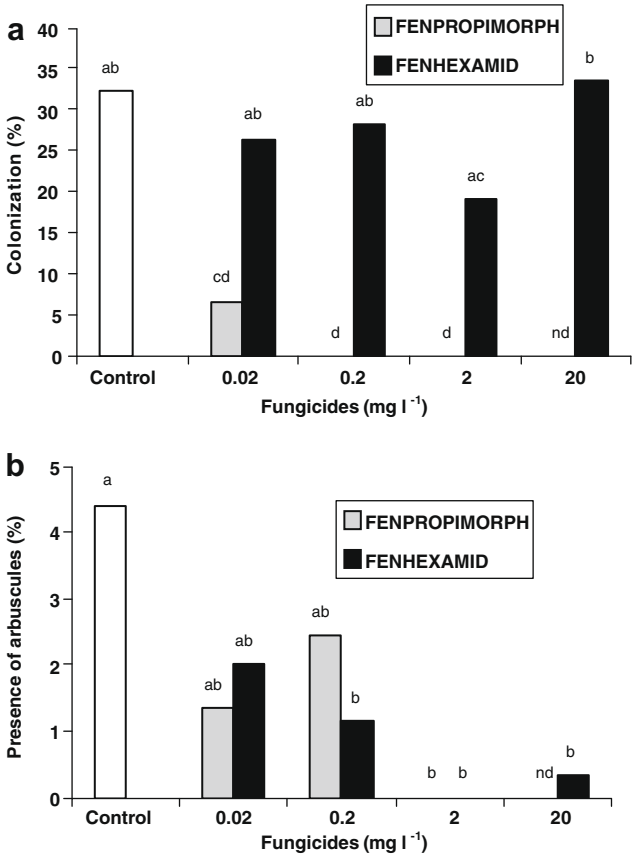

Fig. 3. Estimation of root colonization (a) and presence of arbuscules (b) of carrot roots (*Daucus carota* L.) of *G. intraradices* after 8 weeks of growth in the absence or presence of fenpropimorph or fenhexamid. The means were obtained from four replicates. Data of colonization percentage were converted to arcsine values before the analysis of variance for comparing the mean according to the LSD test ($P < 0.05$). nd: not determinate.

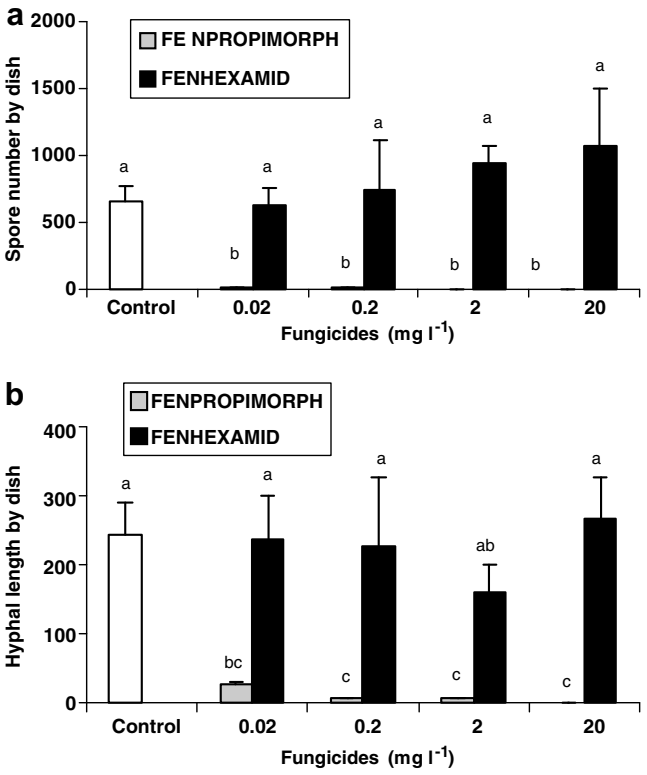

Fig. 4. Effect of different concentrations of fungicides on the spore production (a) and the extraradical hyphae development (b) of *G. intraradices*. Data are presented as mean ± s.e. The mean were obtained from four replicates. Kruskal–Wallis test was applied on sporulation data. Comparisons were performed by Siegel–Castellan test of multiple comparisons ($P < 0.05$). LSD test was applied on hyphal length ($P < 0.05$). Bars sharing the same letters are not significantly different.

deep decrease in spore production was observed with increasing fungicide concentrations. Significant inhibition was also obtained between control and the lowest concentration used. Very few spores (17) were produced with supplemented media (0.02 mg l^{-1} of fenpropimorph) as compared to control where 658 spores were counted. At 20 mg l^{-1} fungicide concentration, 100% of inhibition was observed. On the opposite, no effect of the fenhexamid treatment on sporulation was observed.

The extraradical mycelium production was estimated and presented in Fig. 4. After 8 weeks of incubation, an important inhibition of hyphae development was also observed with fenpropimorph treatments. At the concentration of 0.02 mg l^{-1}, 89% of inhibition was obtained and reached 100% at 20 mg l^{-1}. In contrast, the extraradical fungal development was not disturbed in presence of fenhexamid.

2.4. Fungicide impact on the arbuscular mycorrhizae

In the present study, we clearly demonstrated that two different classes of SBI fungicides such as fenpropimorph and fenhexamid affect totally differently the arbuscular mycorrhizal development when colonization is pre-established.

Fenhexamid showed a total harmless on the vegetal development. But the arbuscule frequency of the symbiotic partner was significantly affected by fenhexamid and not the total colonization level. The absence of sterolic modifications and the only decrease in the total amount of sterols in roots treated with fenhexamid could explain its limited impact on mycorrhization. Influence of this fungicide could be crucial during arbuscule development and then affect the functioning of the symbiosis by limiting exchanges between the partners. Moreover, Zocco et al. (2008) showed that the extraradical fungus and spore production were strongly impacted at high concentrations when the fungus was in direct contact with fenhexamid.

The important toxicity of the fenpropimorph observed on the AM symbiosis could be due to different actions on both partners of the symbiosis.

The first one, fenpropimorph has a very negative impact on the carrot root development related to a sterols content modification. In previous studies, phytotoxic symptoms of fenpropimorph such as growth reduction, necrotic lesions and altered phytosterol composition has been observed in cereals seedlings (Costet-Corio and Benveniste, 1988; Khalil and Mercer, 1991), tobacco calli (Schaller et al., 1991) and *Arabidopsis* (He et al., 2003; Schrick et al., 2004) treated by this fungicide. This phytotoxicity of fenpropimorph seemed not directly related to sterols modification in tobacco tissues (Schaller et al., 1991). But recently, fenpropimorph was observed to cause cell wall abnormalities in dividing root cells which was due to altered sterols composition. These authors demonstrated that sterols were crucial for cellulose synthesis in the building of the cell wall (Schrick et al., 2004). Today there is strong evidence that sterols are essential for normal plant growth (He et al., 2003). In this study, we found that fenpropimorph treated carrot roots exhibited a modified sterols profile (i.e. accumulation of 9β,19-cyclopropylsterols $\Delta^{8,14}$-sterols, Δ^{8}-sterols, and Δ^{7}-sterols) which revealed an inhibition of at least four enzymes of the sterols pathway: cycloeucalenol obtusifoliol isomerase (COI),

$\Delta^{8,14}$-sterol Δ^{14} reductase, Δ^{8}–Δ^{7} isomerase and Δ^{7} sterol C 5(6) desaturase. Indeed, fungicide fenpropimorph has been reported to inhibit these four enzymes (reviewed by Mercer, 1993). Plants treated with this fungicide generally accumulate abnormal 9β,19-cyclopropylsterols and Δ^{8}-sterols at the expense of Δ^{5}-sterols (Costet-Corio and Benveniste, 1988; Grandmougin et al., 1989; Schaller et al., 1991; Cerdon et al., 1996; Hartmann et al., 2002). This indicates that fenpropimorph inhibits the COI which catalyses 9β,19-cyclopropane ring cleavage and also of sterol Δ^{8}–Δ^{7} isomerase. Another observation is the unusual accumulation of Δ^{7}-sterols in fenpropimorph treated carrot roots. This could be consistent with the Δ^{7}-sterol C 5(6) desaturase step inhibition. The last point is the high level of Δ^{8-14}-sterols accumulated in carrot roots treated with fenpropimorph. He et al. (2003) reported a similar acute increase of these abnormal sterols in roots of *Arabidopsis* treated with 10 ppm of fenpropimorph and attributed a toxic effect on the plant development caused by accumulation of these atypical sterols.

The second effect observed with fenpropimorph is the poor development of the fungal symbiotic partner. We observed an important reduction of root colonization, sporulation and extraradical hyphal length. This effect on fungus development could be due to the root alteration. Our study shows that carrot roots treated with fenpropimorph presented deep morphological and sterols content alterations. As major constituents of the plasma membrane, sterols may influence function of membrane bound enzymes involved in transport activity (H^{+}-ATPase) (Grandmougin-Ferjani et al., 1997) or in cellulose synthesis (Schrick et al., 2004). It is logical to assume that there may be cause perturbations in the apoplastic fungal spreading and/or arbuscule functioning.

This study did not allow concluding about a possible direct effect of fenpropimorph on the AM fungus. An impact on extraradical form of this AM fungus was recently demonstrated by Zocco et al. (2008) by the reduction of spore germination dynamics and subsequent germ tube elongation but without affecting spore survival. The biochemical effect of fenpropimorph on the sterol metabolism of *G. intraradices* remains unclear. *G. intraradices* is known to contain a 24-alkylsterol mixture similar to Δ^{5}-sterols detected in plants (Fontaine et al., 2001). In fungi, fenpropimorph sensitive strains accumulated mainly $\Delta^{8,14}$-sterols (Debieu et al., 1992). Nevertheless, no significant increase of these compounds linked to the presence of the arbuscular fungus in roots was detected.

In conclusion, we can say that the two tested fungicides affected differently the mycorrhization. Comparison of induced effects by both fungicides indicates that phytosterols may be implicated in the development of symbiosis. Indeed the modifications of root sterols composition could explain the high fenpropimorph toxicity toward AM symbiosis. On the opposite, absence of sterolic modifications in the roots treated with fenhexamid could account for its more limited impact on mycorrhization.

In the future, it will be interesting to carry out bi-compartmental cultures to study the direct impact of the SBI (especially fenpropimorph) on *G. intraradices* extraradical development in relation with its sterols composition. This will be useful to improve our understanding of the SBI action on non-target organisms.

3. Experimental

3.1. Plant and fungal material

Ri T-DNA-transformed carrot (*Daucus carota* L.) clone DC1, colonized or not by *Glomus intraradices* Schenck and Smith (MUCL 43194), were grown on media MSR (Declerck et al., 1998) modified from Strullu and Romand (1986) solidified with 0.25% (w/v) gellan gel (Phytagel: Sigma, St. Louis, MO, USA) at 27 °C in the dark. Root cultures were started from standardized root inoculum of 4 month old monoxenic cultures Ri T-DNA-transformed carrot, colonized or not by *G. intraradices* as described by Verdin et al. (2006).

3.2. SBI fungicide treatments

Fenpropimorph, and fenhexamid (technical grade) were supplied by BASF AG (Germany), Bayer Crop Science (Germany), respectively. For each experiment, fungicides were dissolved in acetone (5 ml l^{-1} medium), and added to sterilized (121 °C for 15 min) MSR medium (80 °C) in order to obtain, in final concentration, 0.02, 0.2, 2, 20 mg l^{-1} of SBI fungicide or 0.6 μM to 66 μM for the fenpropimorph and the fenhexamid. The similar molarity of the fungicide solutions allow to compare directly fungicides effect by using different mg l^{-1} concentration.

Bottles were shaken by hand and the medium was poured into each standard Petri dish (9 cm). We assumed that most of this solvent was evaporated at the contact of the molten MSR medium as earlier shown by Zohrehvand (2005). Control Petri dishes, containing medium without SBI fungicides, but receiving the same quantity of acetone, were done. In accordance with Wan et al. (1998), we observed that acetone was non-toxic for the AM fungus or host root at this concentration (5 ml l^{-1} medium).

3.3. Effect of SBI fungicides on the growth of colonized or non-colonized carrot roots and the colonization by G. intraradices

Colonized and non-colonized roots by *G. intraradices* were grown in standard Petri dishes containing a 40 ml aliquot of MSR medium supplemented or not by different concentrations of fungicides (0.02, 0.2, 2 and 20 mg l^{-1}). After 8 weeks of culture, roots used as inoculum were eliminated and were not used for morphological data.

After 8 weeks of culture in the dark at 27 °C, the length of roots and hyphae were measured under a dissecting microscope using the gridline intersects technique described by Declerck et al. (2003). The data were integrated in the formula of Newman (1966).

A grid was used and each count was converted to total length using the equation: $R = \pi NA/2\,H$, where N is the number of intersections, A the area within which the root or hyphae lies, H the total length of the straight lines, and R is the total length of roots or hyphae in the Petri dish. Sporulation was also counted using the method described by Declerck et al. (2001).

Roots were collected from media by solubilizing solidified media for 10 min under agitation in 1 volume of Tris buffer (10 mM, pH 7.5).

Mycorrhizal roots were separated from extraradical fungus by filtration on a 0.5 mm sieve. The amount of extraradical adhering to the roots is negligible and can not affect the sterol analysis. Roots were rinsed with sterile water and frozen at −80 °C.

The insufficient growth of roots with 20 mg l^{-1} of fenpropimorph did not allow to perform ulterior analysis (root colonization and sterols analysis).

Root colonization was evaluated. One part of root samples were cleared in KOH (10%) and stained with Chlorazol black E (Brundrett et al., 1994). The magnified intersect method of McGonigle et al. (1990) was used to determine the total AM fungal root colonization and the frequency of arbuscules.

3.4. Sterol extraction, analyses and identification

Before sterols extraction, roots were lyophilised for 48 h. The freeze dried material (20 mg dry wt) was extracted according to protocol described by Costet-Corio and Benveniste (1988). Unsaponifiable fractions, obtained using procedures described by

Grandmougin-Ferjani et al. (1999), were separated on silica gel thin-layer chromatography (TLC) plates ($60F_{254}$; Merck Darstadt, Germany) with dichloromethane as the solvent (two runs). Sterols compounds were visualized under UV (254 nm) after spraying the TLC plates with primuline (0.01% w/v) in aqueous acetone (80%). Bands corresponding to 4,4-dimethyl, 4α-methyl and 4-desmethylsterols fractions migrated at R_f values 0.3; 0.25; 0.15, respectively were scraped off and eluted 30 min with dichloromethane. The silica gel between the R_f of 0.98 and 0.3 was scraped off, eluted with dichloromethane and then analyzed by GC as mentioned above in order to research 3-keto compounds or sterones which were accumulated in *Botryatinia fuckeliana* as described by Debieu et al. (2001). Sterols and their precursors 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 by TLC using dichloromethane (one run). Steryl acetates migrated as a single band which were scraped off and eluted 30 min with dicholoro-methane. Final extracts were analyzed by GC–FID (PerkinElmer, Autosystem, Norwalk, CT, USA) equipped with a glass capillary column EC™-5 (Alltech Associates Inc., Deerfield, IL, USA) (30 × 0.25 mm i.d.) with hydrogen as carrier gas (2 ml min^{-1}). The temperature program included a fast rise from 60 °C to 240 °C at 30 °C min^{-1} and then a rise from 240 to 290 °C at 2 °C min^{-1}, hold for 5 min. Cholesterol was used as the standard for relative retention time (RR_t) determination and quantification of steryl compounds. Identification was done by gas chromatography-mass spectrometry (GC–MS Varian, Walnutcreek, CA, USA) by using specific fragmentation pattern. All the experiments were done in 4 replicates.

3.5. Statistical data analysis

ANOVA analysis was carried out with statistical program STATGRAPHICS release 5.1 (Manugistic, Inc., Rockville, MD, USA). The method used to discriminate between the means was the LSD test ($P < 0.05$). Analysis of variance was conducted on the original data except for the data of colonization and arbuscules percentages which was 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 with the data obtained with the effect of fungicides on sporulation. A non-parametric test 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). All the tests were performed with four replicates per treatments.

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