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Propiconazole Toxicity on the Non-Target Organism, the Arbuscular Mycorrhizal Fungus, *Glomus irregulare*

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that colonize the roots of most terrestrial plants. Indeed, 80% of vegetal species realize this symbiosis (Bonfante and Perotto, 1995). Plants generally benefit from this AMF association through increased plant nutrient uptake, plant growth and survival rates (Smith and Read, 2008). The symbiotic association may also increase host plant resistance/tolerance against biotic (Hol and Cook, 2005; Akhtar and Siddiqui, 2008) and abiotic stresses, including salinity, drought and pollution (Gerdemann, 1968; Franco-Ramirez et al., 2007; Giri et al., 2007; Sudova et al., 2007; Cartmill et al., 2008; Debiane et al., 2008, 2009; Campagnac et al., 2010).

The functioning of AMF may be impaired by cultural practices such as fungicides application (Sukarno et al., 2006). Unfortunately, the use of fungicides is generalized in modern agriculture for the control of fungal diseases. 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 the fungicides, the Sterol Biosynthesis Inhibitor (SBI) family is one of the most 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) Δ14 demethylation or DMIs (e.g. imazalil, prochloraz, triadimenol, propiconazole), (iii) Δ14-reduction and/or $\Delta 8 \rightarrow \Delta 7$ isomerisation (e.g. fenpropidine, fenpropimorph, tridemorph), (iiii) C4 demethylation (e.g. fenhexamid) (Leroux, 2003).

Several studies carried out on SBI fungicide impact on mycorrhizal plants showed contradictory results on the plant growth, on AM fungal development and on the symbiosis functioning (Dodd and Jeffries, 1989; Von Alten et al., 1993; Schweiger and Jacobsen, 1998; Kjoller and Rosendahl, 2000; Schweiger et al., 2001). The use of different experimental procedure in the reported studies (plant species, culture conditions, fungicide formulation, application methods…) did not allow easy comparison with the results obtained and led to some difficulties to give clear conclusion concerning the SBI fungicides effect on AMF (Sancholle et al., 2001).

Monoxenic culture technique has improved noticeably the understanding of the symbiosis (Declerck et al., 2005). The *in vitro* cultures allow non-destructive observations of AMF (Fortin et al., 2002), they are standardized and reproducible method enabling comparisons of various molecules impact on AMF (Debiane et al., 2008, 2009; Hillis et al., 2008; Zocco et al., 2008; Campagnac et al., 2010). In addition, thanks to these monoxenic cultures, big amounts of biological material free of contaminant microorganisms can be obtained.

Zocco et al. (2008) and Campagnac et al. (2008, 2009, 2010) studied the effects of two SBI fungicides, i.e. fenpropimorph and fenhexamid on AMF and root development. The fungitoxicity of fenpropimorph on the development of *Glomus intraradices* was shown by a reduced fungal development, a decrease of sterol content and the increase of a precursor, the squalene suggesting inhibition of an unusual target enzyme in AMF, the squalene epoxidase (Campagnac et al., 2009). Fenpropimorph was also underscored by the significant reduction in root growth and appeared to be due to (i) the replacement of the normal Δ5-sterols by unusual sterols: 9β,19-cyclopropylsterols, Δ8,14-stérols, Δ8-sterols and Δ7-sterols (ii) and the induction of an oxidative stress (lipid peroxidation, antioxidant enzyme activities). However no impact on the sterol profile and root development was detected with fenhexamid (Campagnac et al., 2008), while a significant oxidative stress was highlighted in roots (Campagnac et al., 2010).

Indeed, when plants are subjected to environmental stresses, as SBI fungicides, reactive oxygen species (ROS) are produced, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), alkoxy radical (RO) and the singlet oxygen ($^1O_2$) (Elstner, 1982). The ROS may initiate destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids (Herbinger et al., 2002). As a consequence, higher plants induce efficient antioxidant systems to protect them against oxidative injury (Asada, 1999). The antioxidant systems consist of antioxidant enzymes including superoxide dismustase, peroxidase (POD), catalase, glutathione reductase and non-enzymatic antioxidants including ascorbate and glutathione which are designed to minimize the concentrations of $O_2^{\cdot-}$ and H_2O_2. Nevertheless, antioxidant capacity is dependant on the severity of the stress, on the species and/or on the stage of development (Dat et al., 2000). One of the most damaging oxidative effects is the peroxidation of membrane lipids, which results in the concomitant production of malondialdehyde (MDA), a secondary end product of polyunsaturated fatty acids (FA) oxidation (Hodges et al., 1999; Cho and Park, 2000; Jouili and El Ferjani, 2003). As a response to environmental stress, 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 FA are extremely important to deal with pollutant toxicity and make a contribution to plant survival (Thompson, 1992; Chaffai et al., 2005; Bidar et al., 2008). Modifications in the properties of cellular membranes occur to ensure the function of processes that take within them, and lead to improve growth involving to plant adaptation. Alteration in membrane phospholipids (PL) caused by lipid peroxidation which led to high MDA level is a useful biological marker to highlight the occurrence of oxidative stress conditions (Gallego et al., 1996; Hodges et al., 1999). Whereas a number of studies concerned plant oxidative stress, no study was carried out on the oxidative stress induced by environmental abiotic stress as SBI fungicides on AMF, a beneficial fungus.

In the present work, propiconazole, a SBI belonging to the second group of fungicides (DMIs) and commonly used against mildews and rusts on cereals, was studied.

Propiconazole is a fungicide that inhibits the biosynthesis of ergosterol leading to a decrease capacity of the fungus to maintain normal membrane processes (Köller, 1992). Indeed, in target-fungus, as *Nectria haematococca*, growth inhibition observed with SBI fungicides was correlated with ergosterol biosynthesis inhibition, which led to ergosterol decrease and the accumulation of abnormal or precursor sterols (Debieu et al., 1998). On *Taphrina deformans*, a phytopathogenic fungus, growth inhibition results from an insufficient amount of functional sterol, accumulation of C-14 methyl sterol, or perhaps a combination of both responses to treatment with such an inhibitor (i.e. propiconazole) (Weete et al., 1985).
As propiconazole is a persistent fungicide in environment (half-life estimated to about to 214 days), repeated pulverisations of this SBI could provoke its accumulation in soils (Bromilow et al., 1999). All azole derivative fungicides are directed against cytochrome P450 enzymes. They can have multiple inhibition sites such as the inhibition of sterol formation by blocking 14α-demethylation, gibberellin-biosynthesis or brassinosteroid biosynthesis. Indeed these different metabolims which have many steps thought to be catalyzed by cytochrome P450 monooxygenases (Lupetti et al., 2002; Sekimata et al., 2002). Unfortunately, it could also affect non-target organisms such as plants. Propiconazole phytotoxicity was observed on several occasions (He et al., 1995; Hanson et al., 2003; Meksem, 2007). It seemed that SBI induce perturbations to the hormonal level (gibberellins synthesis) (Rademacher, 1991a,b) and to the photosynthesis level (Gopi et al., 2005). Inhibiting root development, propiconazole could thus have an indirect impact on mycorrhizal fungal development. However, little is known about its potential effect on non-target plant-beneficial fungi such as AMF.
This work was thus focused on investigation of the SBI molecule, propiconazole, toxicity on *Glomus irregulare* development. Moreover, whereas the studies on SBI effect on fungi concerned usually sterol metabolism, oxidative stress was less commonly studied. It is why, we used the monoxenic cultures in order to link up the direct impact of propiconazole, on the AMF *Glomus irregulare* development with the oxidative stress by analysing FA, PL, their associated FA (PLFA) compositions and contents and by measuring malondialdehyde (MDA) production as well as peroxidase (POD) specific activity.

2. Experimental

2.1 Root and fungal growth conditions

The inoculum consisted of Ri T-DNA-transformed chicory roots (*Cichorium intybus* L.) colonized by the AMF *Glomus irregulare*. The AMF used was *Glomus sp.* DAOM 197198 formerly identified as *Glomus intraradices* Schenck and Smith and presently reclassified in a clade that contains the recently described species *Glomus irregulare* Blaszk., Wubet, Renker, and Buscot (Stockinger et al., 2009; Sokolski et al., 2010).
Monoxenic cultures were grown in mono-compartmental Petri dishes (9 cm) on medium 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) without fungicide (control) or amended with different concentrations of propiconazole. A piece of mycorrhizal transformed chicory root was added on medium and the dishes were placed at 27°C in the dark in an inverted position.
Glomus irregulare 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). RC was filled with 25 mL MSR medium without propiconazole and a piece of mycorrhizal transformed chicory roots was added on medium. After 3 weeks,

FC was filled with 30 mL of MSR medium with or without fungicide (control). This compartment was kept root free (by cutting) and used to investigate the treatment effect on extraradical hyphae development in FC. The dishes were also incubated at 27°C in the dark in an inverted position.
For the spore germination test, 50 dishes (5.5 cm diameter) containing 15 mL of MSR medium with or without fungicide (control) were prepared. Spores were extracted from an agar piece containing *Glomus irregulare* spores. This piece of agar was fine-cut to separate spores from hyphae and to facilitate gelose dissolution with Tris buffer (Tris-HCl 50 mM, pH 7.5, EDTA 10 mM). Spores were taken and poured in each 50 dish by treatment (one spore by dish) and dishes were incubated as previously during 30 days.

2.2 Propiconazole treatment

Propiconazole (technical grade) was supplied by Syngenta (Swiss). To prepare fungicide-enriched medium, active matter of propiconazole 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 concentrations of 0.02; 0.2 and 2 $mg.L^{-1}$ of SBI fungicide. The bottles were agitated by hand and the medium was poured into standard mono or bi-compartmental Petri dish (9 cm). Control treatment, containing MSR medium without propiconazole, was supplemented with the same volume of acetone for the mono-compartmental and the FC of the bi-compartmental Petri dishes.

2.3 Fungal growth evaluation

2.3.1 Spore germination

Spore germination on MSR medium with or without (control) propiconazole at 0.02; 0.2 and 2 $mg.L^{-1}$ was followed with low power microscope at 10–40x magnification at 2, 4, 6, 8, 15, 22 and 30 days, to determine the germination percentage of *Glomus irregulare* spores for each treatment. Spores are considered as germinated when a germ tube appears.
Germination rate = Germinated spore number / Total spore number * 100.

2.3.2 Colonization rate

The medium of colonized root cultivated in mono-compartmental Petri dish was solubilized in 1 vol of Tris buffer (Tris–HCl 50 mM, pH 7.5, EDTA 10 mM). The roots were collected by filtration, rinsed with sterile water, cleared in KOH 10% for 1h at 70°C and stained with Trypan Blue (Trypan Blue 0,5 g in 32:32:467 of water:glycerol:lactic acid (v:v:v) for 1h at 70°C (Phillips and Hayman, 1970) to quantify root colonization by *Glomus irregulare*, with the method of McGonigle et al. (1990).

2.3.3 Hyphal length

After 9 weeks of incubation in the mono-compartmental Petri dish, *Glomus irregulare* hyphal lengths were measured under a low power microscope at 10-40x magnification using a gridline intersects technique described by Declerck et al. (2001) and data were integrated using Newman's formula (1966): $L = (\Pi * N * A) / (2 * H)$ (L= hyphal length; N= intersection number; A= gridline surface; H= gridline length sum)
With a little gridline, germ tube resulted from germinated spores were measured as previously after 30 days of incubation.

2.3.4 Spore number

After 9 weeks of incubation with chicory roots, number of spores of *Glomus irregulare* was determined by addition of the number of spores in each 1 cm grid of the gridline used for hyphal length.

2.4 Fatty acid (FA) extraction and analysis

After incubation, *Glomus irregulare* medium was solubilised under agitation in 1 vol Tris buffer (Tris–HCl 50 mM, pH 7.5, EDTA 10 mM) and the fungal mycelium was collected by filtration on a 53 µm sieve, rinsed with sterile water and lyophilized for 48h. Lipids extraction was performed on 3 to 15 mg *Glomus irregulare* dry weight. The material was saponified with 3 mL of 6% (w:v) in methanolic KOH at 70°C for 2h. After addition of 1 vol of distilled water, the saponifiable fractions were extracted three times with 5 vol of hexane and evaporated under N_2. FA were methylated using 3 mL of BF_3:methanol (14%) at 70°C for 3 min, and reaction was stopped in ice. FA methyl esters were extracted three times 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. FA methyl esters were analysed as described in Campagnac et al. (2010) using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionisation detector (Norwalk, CT, USA) and a ECTM- 1000 (Alltech Associates Inc., Deerfield, IL, USA) capillary column (30 m x 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. Their identification relied on the retention times of a wide range of standards (Sigma-Aldrich).

2.5 Phospholipid (PL) extraction and analysis

A second part of fungal samples collected was used to extract PL. PL extraction was carried out as described by Avalli and Contarini (2005). Lyophilized *Glomus irregulare* samples (4 to 14 mg) were dissolved in 20 mL of dichloromethane:methanol (2:1, v:v) at 75°C during 2h to collect all lipid fractions. After filtration and concentration under N_2, lipid fractions were collected in 2 mL of chloroform and applied to Solid Phase Extraction (SPE) cartridges. A silica gel bonded column (GracePure 3 mL volume, 500 mg sorbents, Grace Davidson Discovery Sciences, Alltech, Deerfield, USA) was used. After conditioning with 6 mL of hexane and 3 mL of chloroform:2-propanol (2:1, v:v), lipid fractions were added on the column. Lipid fractions were eluted with 6 mL of the precedent solvent. FA fraction was eluted first with 6 mL of 2% acetic acid in diethyl ether in order to separate them from the PL fractions. Total PL were then collected using two different conditions: (1) with 6 mL of methanol to collect the first part of PL contained in the sample and (2) with 6 mL of chloroform:methanol:H_2O (3:5:2, v:v:v) to recover the rest of phosphatidylcholine which is not totally eluted in the first condition. The recovered fraction was dried under N_2 and was re-dissolved in 0.2 mL of chloroform. A first part (0.1 mL) was injected into HPLC system, the second part (0.1 mL) was collected to extract phospholipid fatty acids (PLFA).

HPLC-ELSD analysis was carried out using an HPLC Waters 600 Controller (Meadows Instrumentation Inc, Bristol, UK) instrument with an automatic injector. A silica analytical column (150 mm x 3.0 mm, i.d. 3 µm) (Alltech, Deerfield, USA) was used. The chromatographic separation was carried out using a linear tertiary gradient according to the following scheme: *t*0 min: 58%A, 40%B and 2%C; *t*7 min: 52%A, 40%B and 8%C and finally *t*22 min: 58%A, 40%B and 2%C. Total chromatography run time 27 min per sample. Eluent

A consisted of 100% isopropanol, eluent B of 100% hexane and eluent C 100% H_2O. The flow rate of the eluent was 1.5 mL.min^{-1}. An Alltech (Deerfield, USA) model 3300 ELSD was used; the pressure of N_2 was maintained at 6 bars and the drift tube temperature was set at 40°C.

2.6 Phospholipid fatty acid (PLFA) extraction and analysis

PLFA collected previously (0.1 mL) were extracted according to the method described above for the total FA extraction and analysis.

2.7 Determination of malondialdehyde (MDA) concentration

A third part of fungal samples collected was used to determine MDA production. To prepare crude cell-free extracts, frozen samples (8 to 30 mg) were ground in mortar with liquid nitrogen. Samples were then suspended in 1 mL of phosphate buffered saline (10 mM). After centrifugation (3 min/10000g), supernatants were divided in 250 µL-aliquots and supplemented with (2,6 di-tert-butyl-4-methylphenol at 2.5 g.L^{-1} ethanol) to determine MDA, peroxidase and proteins. A high performance liquid chromatography MDA assay was used to evaluate the MDA production as described by Debiane et al. (2008). Tetraethoxypropane was used as the standard, and thiobarbituric acid (TBA) as the reagent. 100 µL of either standard solutions or methanol extracts were injected in the HPLC system and the MDA-TBA adducts were detected.

2.8 Determination of peroxidase (POD) activity

One of 250 µL-aliquots collected previously was used to quantify peroxidase (POD) activity. It was measured in supernatants using a commercial available reagent kit as the method described by Mitchell et al. (1994).

2.9 Statistical analysis

Data (spore germination, hyphal length, sporulation, colonization rate, FA contents and percentage, MDA concentration and POD activity) from different concentrations of propiconazole (0; 0.02; 0.2 and 2 mg.L^{-1}) exposed fungus were compared. The means were obtained from five replicates. ANOVA was carried out using Statgraphics release 5.1 (Manugistic, Inc., Rockville, MD, USA). The method used to discriminate between the means was the LSD test ($p<0.05$). Levene's test of variance homogeneity was checked before the use of the multiple comparison procedure. Data of colonization percentages and data of FA percentages were converted to arcsine values before the analysis of ANOVA and LSD test.

3. Results

3.1 Propiconazole decreased drastically *Glomus irregulare* development

3.1.1 Impact of propiconazole on *Glomus irregulare* spore germination

The impact of propiconazole on *Glomus irregulare* spore germination was measured at 2, 4, 6, 8, 15, 22 and 30 days in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}). The obtained spore germination kinetics are presented in Fig. 1. At 2 mg.L^{-1} propiconazole concentration, spore germination was drastically decreased. The reduction was estimated to about 37%, by comparison to the control. Whereas, in the absence of propiconazole (control), maximum of *Glomus irregulare* germination (92%) was reached at day 15, in the presence of 2 mg.L^{-1} of propiconazole, only 58% germination was obtained at the end of the experiment (after 30 days).

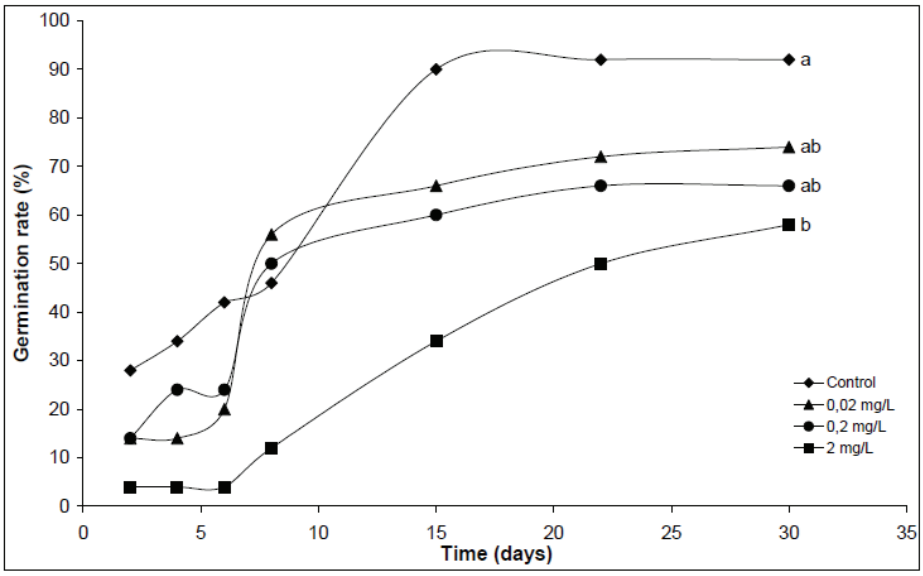

Fig. 1. Spore germination of *Glomus irregulare* at 2, 4, 6, 8, 15, 22 and 30 days in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}). Data are presented as means. The means were obtained from 50 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

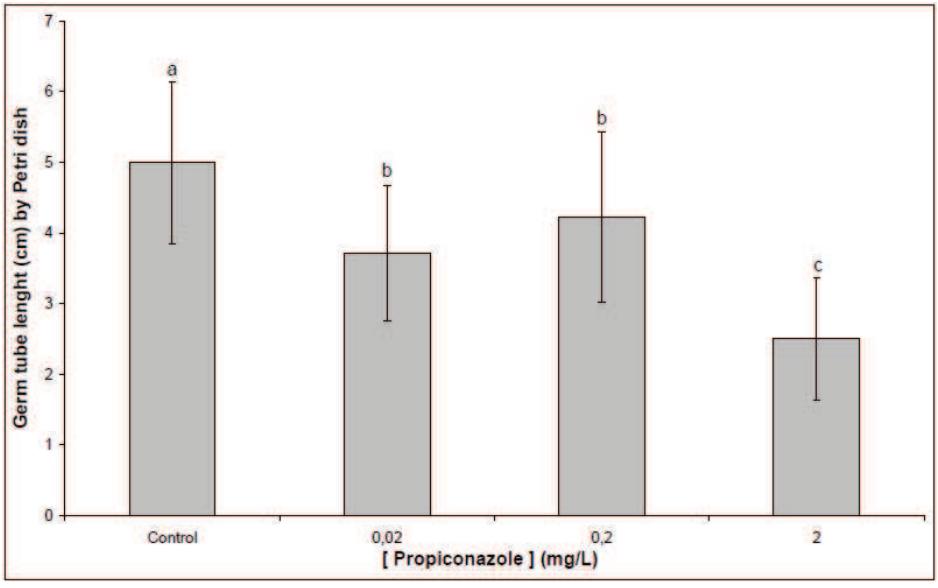

Fig. 2. Germ tube length of *Glomus irregulare* spore after 30 days of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}).

Data are presented as means ± SD. The means were obtained from 22 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

Germ tube lengths of germinative spore grown after 30 days, in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}) are shown in Fig. 2. The length of germ tube which germinated in the absence of propiconazole reached 5 cm. Propiconazole reduced significantly germinative hyphal length from the concentration of 0.02 mg.L^{-1}. A significant decrease in the germ tube length was observed at the highest concentration of the fungicide and was estimated to about 3 cm.

3.1.2 Impact of propiconazole on root colonization by *Glomus irregulare*

The chicory root colonization by *Glomus irregulare* after 9 weeks of incubation in the absence (control) and in the presence of increasing propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) is shown in Fig. 3. The percentage of mycorrhization in chicory roots by *Glomus irregulare* grown without fungicide (control) reached 75% for total colonization, 34% for arbuscules and 36% for vesicles. The total colonization of the chicory roots was significantly decreased in the presence of propiconazole as compared to the control. They were estimated about 59% and 40% respectively at 0.2 and 2 mg.L^{-1}. The arbuscules, also, were significantly reduced by the fungicide. The decreases were about 48% and 68% as compared to the

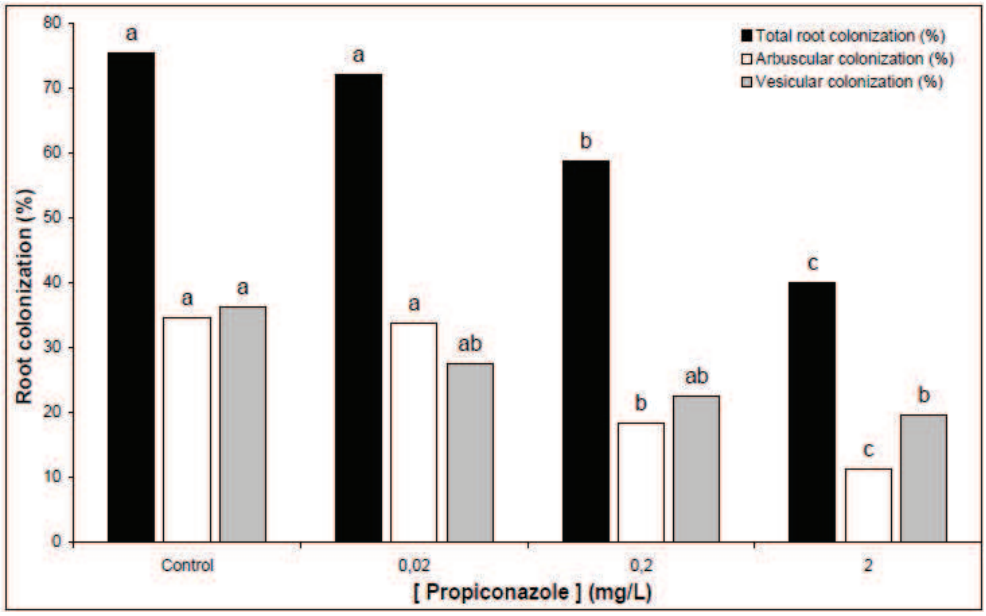

Fig. 3. Chicory root colonization by *Glomus irregulare* (total, arbuscular and vesicular colonization) after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) in mono-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

control in the presence of 0.2 and 2 mg.L^{-1} of propiconazole respectively. Concerning vesicles, significant decrease was observed at the highest concentration of propiconazole (2 mg.L^{-1}) which reach only 19%.

3.1.3 Impact of propiconazole on extraradical hyphae lengths and sporulation of *Glomus irregulare*

The impact of increasing propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) on extraradical hyphae lengths and *Glomus irregulare* spore formation is presented in Fig. 4. In the absence of propiconazole (control), the hyphal length reached more than 860 cm. It decreased significantly on media supplemented with propiconazole from 0.2 mg.L^{-1}.

The hyphal length reached only 397 and 118 cm in the presence of 0.2 and 2 mg.L^{-1} propiconazole respectively. *Glomus irregulare* sporulation decreased significantly from 0.2 mg.L^{-1}. Reduction was about 29% as compared to the control. At the highest propiconazole concentration (2 mg.L^{-1}), the spore number was drastically reduced and was estimated only to 35 spores by dish, whereas the control reached 2901 spores.

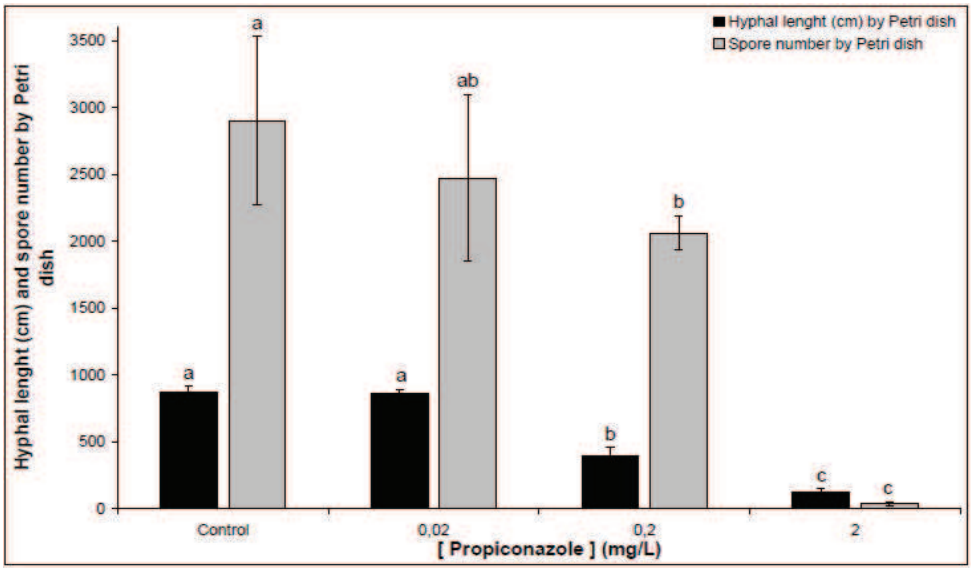

Fig. 4. Hyphal length and sporulation of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in mono-compartmental Petri dish. Data are presented as means. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

3.2 Propiconazole disturbed *Glomus irregulare* FA content

Glomus irregulare FA compositions and contents, when growing without and with increasing propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) in bi-compartmental Petri dish, are shown in Table 1. It was found that the distribution of *Glomus irregulare* FA

Fatty acids (FA)	Control		[Propiconazole] (mg.L^{-1})							
			0.02		0.2		2			
	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%
C16:0	44.27 ± 4.16^{a}	12^{a}	46.97 ± 4.87^{a}	12^{a}	47.57 ± 7.74^{a}	12^{a}	42.98 ± 6.56^{a}	11^{a}
C16:1ω5	283.83 ± 32.49^{b}	76^{a}	300.03 ± 39.92^{b}	76^{a}	295.11 ± 49.26^{b}	74^{a}	289.02 ± 40.75^{b}	76^{a}
C18:0	0.15 ± 0.02^{a}	0.04^{a}	0.13 ± 0.03^{a}	0.03^{a}	0.2 ± 0.04^{a}	0.05^{a}	0.29 ± 0.1^{a}	0.07^{a}
C18:1	18.7 ± 2.54^{a}	5^{a}	20.15 ± 2.46^{a,b}	5^{a}	25.53 ± 4.22^{b}	6^{a}	22.87 ± 2.97^{a,b}	6^{a}
C18:2	2.42 ± 0.31^{a}	0.6^{a}	2.47 ± 0.27^{a}	0.6^{a}	2.6 ± 0.41^{a}	0.6^{a}	2.51 ± 0.4^{a}	0.6^{a}
C19:0	3.09 ± 0.44^{a}	0.8^{a}	3.3 ± 0.43^{a}	0.8^{a}	3.54 ± 0.59^{a}	0.9^{a}	3.55 ± 0.5^{a}	0.9^{a}
C18:3	0.13 ± 0.02^{a}	0.03^{a}	0.14 ± 0.03^{a}	0.04^{a}	0.16 ± 0.03^{a,b}	0.04^{a}	0.19 ± 0.03^{b}	0.05^{a}
C20:1	0.42 ± 0.05^{a}	0.1^{a}	0.46 ± 0.07^{a}	0.1^{a}	0.42 ± 0.15^{a}	0.1^{a}	0.41 ± 0.09^{a}	0.1^{a}
C20:2	0.53 ± 0.11^{a}	0.1^{a}	0.56 ± 0.07^{a}	0.1^{a}	0.6 ± 0.12^{a}	0.1^{a}	0.85 ± 0.29^{b}	0.2^{a}
C21:0	8.24 ± 1.06^{a}	2^{a}	8.86 ± 1.08^{a}	2^{a}	9.29 ± 1.59^{a}	2^{a}	9.15 ± 1.2^{a}	2^{a}
C20:3	2.6 ± 0.39^{a}	0.7^{a}	2.67 ± 0.35^{a}	0.7^{a}	2.69 ± 0.52^{a}	0.7^{a}	2.73 ± 0.35^{a}	0.7^{a}
C20:5	1.1 ± 0.16^{a}	0.3^{a}	1.22 ± 0.22^{a}	0.3^{a}	1.4 ± 0.39^{a}	0.3^{a}	1.17 ± 0.21^{a}	0.3
C22:0	4.39 ± 0.61^{a}	1^{a}	4.81 ± 0.61^{a}	1^{a}	4.93 ± 0.87^{a}	1^{a}	4.98 ± 0.8^{a}	1^{a}
C22:1	0.82 ± 0.09^{a}	0.2^{a}	0.82 ± 0.25^{a}	0.2^{a}	0.96 ± 0.48^{a}	0.2^{a}	1.03 ± 0.22^{a}	0.3^{a}
C22:2	0.14 ± 0.04^{a}	0.04^{a}	0.16 ± 0.02^{a}	0.04^{a}	0.3 ± 0.11^{b}	0.07^{a}	0.24 ± 0.08^{a,b}	0.06^{a}
Total FA (mg.g^{-1} dry weight)	370.83 ± 38.56^{a}		392.78 ± 50.09^{a}		395.05 ± 64.82^{a}		381.98 ± 53.75^{a}	
Saturated/unsaturated FA	0.19^{a}		0.19^{a}		0.2^{a}		0.19^{a}	

Table 1. Fatty acid (FA) composition and content of *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish.
Data are presented as means ± SD. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

ranged from C16:0 to C22:2 with three major compounds (C16:0, C16:1ω5 and C18:1). *Glomus irregulare* FA profile was not affected by the presence of the SBI fungicide propiconazole. The total FA contents and the ratio saturated/unsaturated FA of *Glomus irregulare* were similar when the AMF was grown in propiconazole supplemented medium or not. However, the amounts of unsaturated FA were modified by the treatment. The FA C18:1 and C22:2 increased significantly by about 36% and 114% respectively from 0.2 mg.L^{-1} of propiconazole and the FA C18:0, C18:3 and C20:2 increased at the highest concentration (2 mg.L^{-1}) by about 93%, 46% and 60% respectively.

3.3 Propiconazole affected phosphatidylcholine content of *Glomus irregulare* and its associated FA

Phospholipid content analysis of *Glomus irregulare* after 9 weeks of incubation showed that the presence of propiconazole at the concentration of 2 mg.L^{-1} increased significantly phosphatidylcholine quantity by about 207% as compared to the control (Fig. 5.).

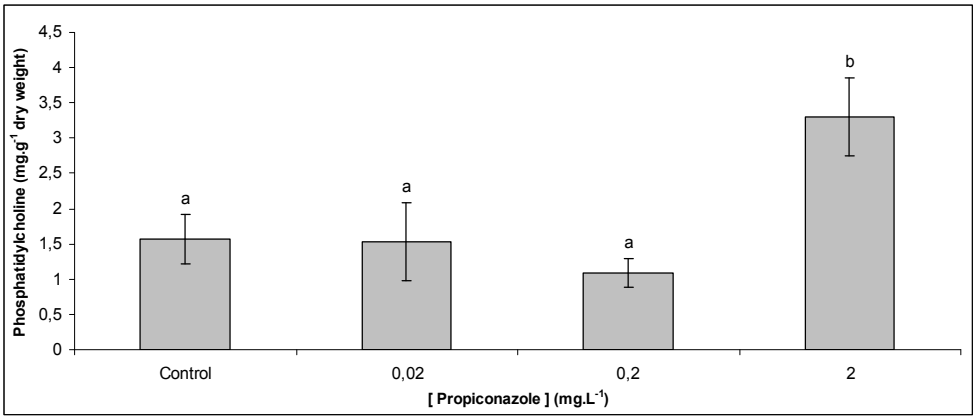

Fig. 5. Phosphatidylcholine content of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± standard error. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

Glomus irregulare phospholipid fatty acids (PLFA) compositions and contents, when growing without and with increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}) in bi-compartmental Petri dish, are shown in Table 2. Six PLFA were detected: C16:0 as the major constituent, C16:1ω5, C18:0, C18:1, C18:3 and C20:2. No significant differences were found in the proportion of each PLFA as compared to the control. But the ratio saturated/unsaturated PLFA and the quantity of each PLFA increased at the highest propiconazole concentration (2 mg.L^{-1}), except for the PLFA C16:1ω5, C20:2 and C18:1 which was not detected at this concentration. In addition, the quantity of total PLFA increased at the highest propiconazole concentration (2 mg.L^{-1}).

	Control		[Propiconazole] (mg.L^{-1})					
			0.02		0.2		2	
Phospholipid fatty acids (PLFA)	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%
C16:0	0.49 ± 0.12^{a}	54^{a}	0.47 ± 0.12^{a}	51^{a}	0.47 ± 0.1^{a}	51^{a}	1.23 ± 0.43^{b}	57^{a}
C16:1ω5	0.07 ± 0.02^{a}	8^{a}	0.1 ± 0.05^{a}	10^{a}	0.14 ± 0.09^{a}	14^{a}	0.14 ± 0.06^{a}	6^{a}
C18:0	0.11 ± 0.03^{a}	12^{a}	0.12 ± 0.05^{a}	12^{a}	0.11 ± 0.01^{a}	12^{a}	0.34 ± 0.18^{b}	16^{a}
C18:1	0.03 ± 0.03^{a}	3^{a}	0.06 ± 0.06^{a}	5^{a}	0.02 ± 0.01^{a}	3^{a}	n.d.	
C18:3	0.12 ± 0.02^{a}	14^{a}	0.09 ± 0.03^{a}	10^{a}	0.12 ± 0.04^{a}	13^{a}	0.25 ± 0.05^{b}	12^{a}
C20:2	0.09 ± 0.08^{a}	9^{a}	0.14 ± 0.09^{a}	12^{a}	0.06 ± 0.04^{a}	7^{a}	0.21 ± 0.13^{a}	9^{a}
Total PLFA (mg.g^{-1} dry weight)	0.91 ± 0.0.24^{a}		0.98 ± 0.35^{a}		0.92 ± 0.23^{a}		2.17 ± 0.82^{b}	
Saturated/ unsaturated PLFA	1.93^{a}		1.51^{a}		1.71^{a}		2.45^{b}	

Table 2. Phospholipid fatty acids (PLFA) composition and content of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from 5 replicates. different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$). n.d.: not detected.

3.4 Propiconazole induced oxidative stress in *Glomus irregulare*

Malondialdehyde (MDA) production (a lipid peroxidation biomarker) and peroxidase (POD) specific activities (an anti-oxidant enzyme) in *Glomus irregulare*, when growing in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}) are shown in Table 3. Significant increases in the MDA content were pointed

[Propiconazole] (mg.L^{-1})	MDA (µmol.g^{-1} of protein)	POD activity (nKat.g^{-1} of protein)
Control	0.775 ± 0.224^{a}	2.8E-08 ± 1.8E-08^{a}
0.02	1.334 ± 0.19^{ab}	2.4E-08 ± 1.5E-08^{ab}
0.2	1.611 ± 0.674^{b}	2.2E-08 ± 1.6E-06^{ab}
2	1.559 ± 0.583^{b}	5.7E-09 ± 2.7E-09^{b}

Table 3. Concentration of malondialdehyde (MDA) and peroxidase (POD) activities in *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from five replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.02$).

out in *Glomus irregulare* treated with propiconazole at 0.2 and 2 mg.L^{-1}. These rises were estimated to about 108% and 101% respectively.
In the absence of propiconazole, POD specific activities were estimated to about 2.8E^{-08} nKat.g^{-1} of protein. It decreased significantly nearly 80% at the highest concentration of propiconazole, to reach 5.7E^{-09} nKat.g^{-1} of protein.

4. Discussion

In the present work, we have pointed out the toxicity of the SBI molecule, propiconazole, on the non-target fungus, *Glomus irregulare*. The study focused on the effect of the fungicide on the FA, PL and PLFA compositions and contents, lipid peroxidation (evaluated in term of MDA content), and antioxidant enzyme activities (evaluated through the determination of POD activity), in relation with the AMF development.
Glomus irregulare development has been shown to be negatively impacted by increasing propiconazole concentrations (0.02, 0.2 and 2 mg.L^{-1}). Drastic reductions have been observed in the main steps of the AMF life cycle (germination, hyphal elongation, root colonization, extra-radical hyphae development and spore production). Contradictory effects of different propiconazole formulations applied on AMF colonized plants grown in pots were observed. No negative impacts on AMF development were reported by Nemec (1985), Hetrick et al. (1988), Plenchette and Perrin (1992), Von Alten (1993) and Kjoller and Rosendahl (2000). However inhibition of plant colonization and spore germination were described (Dodd and Jeffries, 1989; Plenchette and Perrin, 1992). The diversity of experimental procedures of these studies (plant species, growth conditions, fungicide formulations, application methods of fungicides, micro-organisms) present led to some difficulties to compare results, and to conclude on the impact of SBI on AMF (Sancholle et al., 2001). In our study, we used monoxenic cultures which are easily reproducible and used by different authors in order to evaluate toxicity of fungicides on AMF (Wan et al., 1998; Campagnac et al., 2008, 2009; Zocco et al., 2008). Inhibitory effects on fungal development of two other SBI fungicides i.e. fenpropimorph and fenhexamid was obtained in this way (Campagnac et al., 2008, 2009; Zocco et al., 2008).
Inhibition of spore germination could induce negative consequences on root colonization and fungi surviving. Moreover, as the main role of the mycorrhizal symbiosis is to improve the uptake of soluble mineral elements as phosphorus and nitrogen, present in soil in lower concentrations, the depletion of the AM colonization, in particular the arbuscular colonization in the presence of propiconazole, as shown in our data, could thus have a negative impact on plant water and mineral nutrition and on plant health. The negative propiconazole effect on root colonization and phosphate uptake by the AMF were reported by Dodd and Jeffries (1989) and Hetrick et al. (1988) respectively. Besides fungicides, many studies reported negative effects on *Glomus sp.* development of various pollutants (i.e., polycyclic aromatic hydrocarbons and heavy metal) and other abiotic stresses (i.e., salinity) (Schützendübel and Polle, 2002; Verdin et al., 2006; He et al., 2007; Hildebrandt et al., 2007; Debiane et al., 2008, 2009).
In order to explain propiconazole toxicity on AMF development, changes in the lipids, especially FA, PL, PLFA and the lipid peroxidation biomarker MDA, were investigated in *Glomus irregulare* grown under propiconazole treatment.

The composition of *Glomus irregulare* FA is ranged from C16:0 to C22:2 with three main compounds C16:1ω5 as major FA, C16:0 and C18:1. This FA profile is in agreement with previous studies carried out on different *Glomus sp.* (Gaspar et al., 1994; Fontaine et al., 2001; Sancholle et al., 2001; Grandmougin-Ferjani et al., 2005). Whereas propiconazole did not affect the AMF total FA contents, FA composition and the proportion of each FA, significant increases of *Glomus irregulare* unsaturated FA C18:1, C18:3, C20:2, C22:2 contents were observed while the treatment was applied as compared to the control.

In addition, our data showed a drastic increase of phosphatidylcholine at the highest propiconazole concentration. This result is in accordance with the study of Weete et al. (1985) which reported an increase in PL plasma membrane of the pathogenic fungus, *Taphrina deformans* in presence of propiconazole at a concentration which inhibited its growth by 50%. Moreover, in presence of propiconazole at 2 $mg.L^{-1}$, PLFA analysis highlighted significant increases in the total quantity due mainly to C16:0, C18:0 and C18:3 increases. Similar disturbances in PL and in PLFA quantities were also described in plant under salinity stress (Parti et al., 2003; Elkahoui et al., 2004). Parti et al. (2003) explained these lipid changes by mean of adaptations which increase the ability of the plant to endure salinity. Thus, increases of phosphatidylcholine and PLFA quantities observed in *Glomus irregulare* grown under propiconazole treatment could indicate some possible adaptations of the AMF under the SBI fungicide stress in order to try to maintain its membrane integrity and fluidity compatible with an optimal membrane functionality. Indeed, our results showed that the saturated/unsaturated FA ratio was more important in the presence of propiconazole at 2 $mg.L^{-1}$. This ratio saturated/unsaturated FA increase suggested a modification in membrane composition and is in agreement with the study of Benyagoub et al. (1996) which reported an increase in membrane fluidity on *Fusarium oxysporum* when exposed to an antifungal compound. In the same way, Kohli et al. (2002) found an increase of membrane fluidity in *Candida albicans* treated with azoles.

The disturbance in the unsaturated FA, especially the polyunsaturated C18:3, in the presence of propiconazole reminds an induction of lipid peroxidation. Indeed, our results pointed out concomitant increases of MDA production in *Glomus irregulare* under propiconazole treatment suggesting an oxidative stress. The disruption in polyunsaturated FA levels may therefore be related to the direct reaction of oxygen-free radicals with unsaturated lipids. These observations are in accordance with previous studies, which reported that abiotic stresses such as pollution, drought, salinity and heat induced an oxidative stress leading to MDA production by plant cells (Sinha et al., 2005; Bidar et al., 2007; He et al., 2007; Debiane et al., 2008, 2009; Yamauchi et al., 2008). In fact, the oxidative stress arising from abiotic stress exposure could generate ROS (Apel and Hirt, 2004), which can interact with polyunsaturated FA to generate aldehydes of which MDA is the main one (Esterbauer et al., 1991). The increase of MDA content in the AMF in presence of propiconazole, suggested an oxidative stress, which can be involved in mediating compositional membrane disruption, demonstrated in our conditions by important increases in phosphatidylcholine and its associated FA.

Little is known about ROS scavenging systems in AMF. To date only genes encoding three proteins putatively involved in ROS homeostasis have been identified and characterized in AMF: a CuZn superoxide dismutase in *Gigaspora margarita* (Lanfranco et al., 2005) and six genes putatively encoding glutathione S-transferases (GST) (Waschke et al., 2006) and a

metallothionein (González-Guerrero et al., 2007) in *Glomus intraradices*. Although the metallothionein was potentially involved in metal chelation, it was also shown to be involved in ROS scavenging, an activity that results from the capability of their thiolate groups to be reversibly oxidized (González-Guerrero et al., 2007). Recently, Benabdellah et al. (2009), provided the first evidence for the existence of a functional glutaredoxin (GintGRX1) in the AM fungus *Glomus intraradices*, a multifunctional protein with oxidoreductase, peroxidase and GST activity. Their findings also indicated that GintGRX1 might play a role in oxidative stress protection in the AM fungus. Antioxidant enzymes are important components in preventive oxidative stress. In the present study, POD specific activity was found to be inhibited at the highest concentration of propiconazole (2 $mg.L^{-1}$) indicating that the AMF antioxidant capacity were reduced when *Glomus irregulare* was grown on media containing propiconazole. Whereas fungicides as fenpropimorph and propiconazole induced an increase in plant POD activity (Jaleel et al., 2008; Campagnac et al., 2010), indicating an enhancement of free radicals under fungicide stress, our study showed a depletion of the antioxidant enzymes production in *Glomus irregulare* under propiconazole treatment. These results indicated that propiconazole stress could have a negative effect on proteins such as POD and that could thus damage the ROS scavenging by antioxidant enzymes.

To our knowledge, this is the first study which concerns the direct impact of propiconazole on the FA metabolism of the AMF *Glomus irregulare* in relation with its development. Taken in their whole, our data suggest that the drastic decrease of *Glomus irregulare* development observed could be linked to lipid (total FA, PL and PLFA) metabolism perturbation and to the toxicity of MDA accumulation. MDA production originated from the peroxidation of membrane lipids which could affect membrane functionality and consequently its function in nutrient uptake, exchanges, signal transmission and membrane enzyme regulation. According to these results, there will be probably a relationship between fungicide toxicity, the production of ROS under fungicide stress, lipid peroxidation, and membrane function disturbance, very probably due to an alteration of the membrane composition. It could be interesting, in a future work, to consider the target-lipid classes that also constitute the membrane (i.e. sterols). Moreover, a study of lipid rafts, functional microdomain of plasma membrane could be particularly interesting.

In conclusion, this study presented herein has contributed not only to investigate the toxicity of agricultural chemicals on AMF but also can provide a useful approach in soil ecotoxicology studies and risk assessment. In addition, the data highlighted the importance of investigating for side effects of pesticides on non-target soil organisms and demonstrated the emergency of using sustainable alternative method to control plant diseases. This work further emphasized the interest of *in vitro* cultures to investigate the mechanisms behind the impact of disease control molecules on the non-target AM fungal symbionts.

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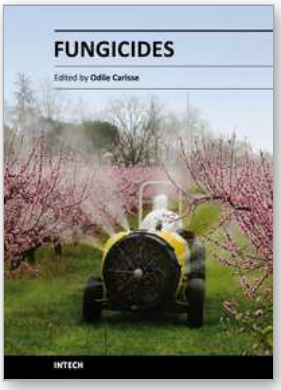

Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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