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Is the arbuscular mycorrhizal fungus *Rhizophagus irregularis* able to fulfil its life cycle in the presence of diesel pollution?

Sihem Driai [a, b], Anthony Verdin [a], Frédéric Laruelle [a], Arifa Beddiar [b], Anissa Lounès-Hadj Sahraoui [a, *]

[a] Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), Université du Littoral Côte d'Opale (ULCO), 50 Rue Ferdinand Buisson, BP 699, 62228 Calais Cedex, France
[b] Laboratoire de Biologie Végétale et Environnement, Université Badji Mokhtar, BP 12, 23000 Annaba, Algeria

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ABSTRACT

The present work examined the impact of increasing diesel concentrations (0.05, 0.1, 0.25, 0.5 and 1%) on the development of both partners of the arbuscular mycorrhizal symbiosis: *Rhizophagus irregularis* and chicory roots (*Cichorium intybus* L.) grown *in vitro*. Our findings showed that although the different diesel concentrations tested (0.05, 0.1, 0.25, 0.5%) affect negatively the main stages of *R. irregularis* development (germination, germinative hyphal elongation, root colonization rate, extraradical hyphae development, sporulation) and the chicory root growth, they are not completely inhibited, except at 1%. The arbuscular mycorrhizal fungus was able to fulfil its life cycle in the presence of the pollutant. No increase in malondialdehyde (MDA) production – a biomarker of lipid peroxidation – was detected in diesel-exposed mycorrhizal or non-mycorrhizal roots, suggesting that the negative effect of diesel on the chicory roots growth could not result from the alteration of membrane lipids. Moreover, our results pointed out that the diesel toxic effect on the growth of chicory roots is less noticeable when they are mycorrhized, indicating a protective effect of mycorrhization. This protection could be related to induction of antioxidant enzyme peroxidase activity, but not to superoxide dismutase activity. Taken together, our results demonstrated the toxic effect of diesel on the mycorrhizal symbiosis and suggest a probable involvement of the mycorrhizal fungus in the protection of chicory roots against oxidative stress induced by diesel.

1. Introduction

Diesel is a hazardous fuel commonly used for vehicles and machinery. Its composition is very varied and dependent on its origin and on current legislation. It is a complex mixture of petroleum hydrocarbons (HC) with an average carbon number of C8–C26. The majority of its components consist of alkanes, both straight chained and branched and aromatic compounds including mono-, di- and polyaromatic hydrocarbons, which are relatively persistent in the soil (Adam and Duncan, 1999). The low molecular weight of diesel HC compounds, make of it more toxic than some of other petroleum products because the low molecular weight compounds are more soluble and bioavailable than high molecular weight ones (Kauppi et al., 2011). The increased use of diesel may cause a permanent risk to the environment and to humans (Abed et al., 2002). The presence of diesel HC in the environment not only adversely affects human health but also plant growth and development. Diesel hydrocarbons hinder the growth of plants, the multiplication of many other microorganisms and the microbiological processes in soil.

The toxic effect of organic pollutants on plants and soil microorganisms has been demonstrated by several authors (Leyval and Binet, 1998; Verdin et al., 2006; Debiane et al., 2008, 2009; Campagnac et al., 2010; Hernández-Ortega et al., 2012). Organic pollutants can limit plant growth and mineral nutrient absorption (Merkl et al., 2005; Hernández-Ortega et al., 2012). They can act directly by inhibiting seed germination and seedling root growth (Adam and Duncan, 1999; Maila and Cloete, 2002) and reducing photosynthetic rates (Macfie and Taylor, 1992), or indirectly by

Abbreviation: AMF, arbuscular mycorrhizal fungi; SOD, superoxide dismutase; POD, peroxidase; MDA, malondialdehyde; ROS, reactive oxygen species.

* Corresponding author.
E-mail addresses: driai_sihem@yahoo.fr (S. Driai), lounes@univ-littoral.fr (A. Lounès-Hadj Sahraoui).

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changing soil chemical parameters (Kisic et al., 2009) and affecting soil microorganisms activities (Lapinskiene et al., 2006; Barrutia et al., 2011; Guo et al., 2012). Moreover, organic pollutants induce the formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\bullet}$) (Parida et al., 2004). Among the oxidative damages caused by ROS, there is peroxidation of membrane lipids that can generate malondialdehyde (MDA) used as a biomarker of membrane alterations (Bailly et al., 1996; Arora et al., 2002; Goel and Sheoran, 2003). Plants have the ability to maintain a non-toxic level of ROS using non-enzymatic (Dat et al., 2000) and enzymatic antioxidant systems such as superoxide dismutase (SOD) which catalyses the dismutation of $O_2^{\cdot-}$ to H_2O_2, and peroxidase (POD) which decomposes H_2O_2 (Arora et al., 2002).

To overcome the detrimental effects and to improve plant tolerance to stresses, the plants adopt several strategies including the development of symbiosis with arbuscular mycorrhizal (AM) fungi. Present in the soils of most ecosystems, AM fungi form symbiotic associations with the roots of over 80% of the terrestrial plant species (Smith and Read, 2008). Through its mycelium network, mycorrhizal symbiosis improves plant water and nutrient uptake especially phosphorus (Schreiner, 2007; Mardukhi et al., 2011; Hernández-Ortega et al., 2012; Labidi et al., 2012). Arbuscular mycorrhizal fungi also play a role in increasing plant tolerance to abiotic stresses such as pollutants (Leyval et al., 2002; Alarcón et al., 2006; Verdin et al., 2006; Debiane et al., 2009), and biotic stresses such as plant pathogens (Dalpé, 2005; Akhtar and Siddiqui, 2008). It was also demonstrated that AM fungi can facilitate plant establishment and survival in hydrocarbons-contaminated soils and also contribute to increase hydrocarbon biodegradation in the plant rhizosphere through stimulation of soil microbial communities with xenobiotic degradation abilities (Binet et al., 2000; Zhou et al., 2013; Nwoko, 2014). However, little is known about the impact of organic pollutants such as diesel on AM fungi development.

Thus, this work aims to study, in monoxenic conditions, the impact of increasing diesel concentrations on the main stages of the AM fungus *Rhizophagus irregularis* development (spore germination, germinative hyphae elongation, root colonization rate, extraradical hyphae development and sporulation) and on the growth of its host, chicory roots (elongation and dry biomass). At the biochemical level, oxidative stress biomarkers such as the production of MDA as well as SOD and POD enzyme activities are assessed.

2. Material and methods

2.1. Plant and fungal material

R. irregularis MUCL 43194 (DAOM 197198) (Schüßler and Walker, 2010) spores were used in the first experiment (spore germination kinetics). Chicory root cultures (*Cichorium intybus* L.), transformed with *Agrobacterium rhizogenes* and colonized or not by the same AM fungus *R. irregularis*, were undertaken in the second experiment (*in vitro* culture of mycorrhizal and non-mycorrhizal chicory roots).

2.2. Preparation of the culture medium

The culture medium used is the modified M medium (Bécard and Fortin, 1988) solidified with 0.3% (w v-1) gellan gel (Phytagel; Sigma, St. Louis, MO, USA) and autoclaved at 120 °C for 20 min. The diesel was filter sterilized at 0.25 μm in a horizontal flow hood and added to the modified M medium in order to obtain the concentrations of 0.05, 0.1, 0.25, 0.5 and 1%. The control used in this experiment is the M medium without diesel (0% diesel). The diesel has a cetane number of 51 and a total polycyclic aromatic hydrocarbons amount less or equal to 8.0% (w/w).

Addition of diesel at the concentration of 0.05% did not change the pH value of the M medium which was equal to 5.9. A slight decrease of 0.01 and 0.02 unit in the pH values was measured at the 0.1 and 0.25% diesel concentrations respectively when compared with the control. At 0.5 and 1% diesel concentrations, the pH culture medium values decreased from 5.9 to 5.63 and 5.42 respectively.

2.3. Methods

2.3.1. Germination of R. irregularis spore experiment

Spores were extracted from the fungal compartment of a 2-month-old monoxenic culture of Ri T-DNA transformed chicory (*C. intybus* L.) roots colonized by *R. irregularis*. One spore was placed in the middle of each Petri dish (5.5 cm diameter) containing 10 mL of the M medium supplemented or not (Control) with the different diesel concentrations (0.1, 0.25, 0.5 and 1%). The dishes were incubated for 30 days at 27 °C in the dark.

2.3.1.1. Determination of R. irregularis spore germination kinetics and germination hypha development. The number of germinated spores was counted under an optical microscope (GX100) after 3, 5, 7, 10, 20 and 30 days of incubation. A spore is considered as germinated when a germ tube is observed. After 30 days of incubation, the hyphal length was measured under an optical microscope using the method of Newman (1966), and the number of spores germinating in a branched or linear mode was counted.

2.3.1.2. Spore viability test. Non-germinated spores were collected and then rinsed with sterile distilled water and transferred onto a diesel free medium. The spore cultures were incubated during one week at 27 °C in the dark. The germinated spores were counted under an optical microscope (GX100).

2.3.2. In vitro culture of chicory root colonized or not with R. irregularis experiment

Mycorrhizal and non-mycorrhizal chicory roots transformed by *A. rhizogenes* (Fontaine et al., 2004) were grown in a standard mono-compartmental Petri dishes (9 cm diameter) containing 25 mL aliquot of the modified M medium supplemented or not (control) with increasing diesel concentrations (0.05, 0.1, 0.25, 0.5 and 1%). A piece of agar (1.5 cm^2;) of 2-month-old mycorrhizal or non-mycorrhizal chicory root was placed on the middle of each Petri dish. Five dishes per treatment were prepared and incubated in an inverted position during 9 weeks at 27 °C in the dark.

2.3.2.1. Determination of fungal and root growth. After 9 weeks of incubation, the hyphal lengths were measured using the method described by Declerck et al. (2003) which consists in counting the number of intersections of the roots on a circular gridlines and applying the formula of Newman (1966). Root and extraradical hyphal lengths were determined using the same methods described for the determination of hyphal lengths. The number of spores formed was determined by adding the number of spores recorded in each cell of the circular gridlines (Declerck et al., 2001). The roots were collected after solubilising the culture media during 15 min under agitation in 25 mL of Tris–HCl buffer (50 mM, pH 7.5) + EDTA (10 mM) (v/v), and collected by filtration on a 0.5 mm sieve. Roots were then rinsed with distilled water. A first aliquot of the mycorrhizal roots collected from each replicate were cleared in a solution of KOH 10% (w v-1) and stained with 0.05% blue trypan in lactic acid as described by Phillips and Hayman (1970) to determine arbuscular mycorrhizal colonization using the gridline intersect

method of McGonigle et al. (1990). The second aliquot of mycorrhizal and non-mycorrhizal roots collected from each replicate were lyophilized during 48 h and weighed to determine the dry weight.

2.3.2.2. Preparation of root extract. Between 8 and 30 mg of the non mycorrhizal and mycorrhizal chicory roots was ground to a fine powder in liquid nitrogen with a mortar and pestle. The powdered roots were suspended in 1 mL of phosphate buffer (10 mM). After centrifugation (1000g for 3 min) at 4 °C, 2.5 μL of 2.6 di_tert-butyl-4-methylphenol (at 2.5 g L−1 ethanol) was added to 250 μL of the supernatant to prevent the MDA oxidation. The rest of the supernatant was used to determine the protein content and the total activity of SOD and POD enzymes.

2.3.2.3. Determination of MDA concentration. The production of MDA was assessed by high performance liquid chromatography (HPLC) (Shirali et al., 1994). 200 μL of root aliquot were mixed with 1 mL of HCl (0.1 N) and extracted twice with 3 mL of ethylacetate. The mixture was stirred for 5 min and centrifuged at 3000g for 10 min. The organic layer was collected and evaporated under the stream nitrogen. After evaporation, the extract was suspended in 100 μL of methanol. The HPLC system of Jasco PU-980 pump, was equipped with a Nucleosil column (C18, 150 × 4.6 mm, 5 μm particle size), a Rheodyne 7725 automated injector, a UV detector (detection wavelength = 532 nm) and a Shimadzu CR3A integrator (Vasse Industries, Lille, France). The mobile phase was a blend of 50 mM KH_2PO_4 and methanol 60:40 (v v-1) adjusted to pH 6.8 (KOH 1 M). Tetraethoxypropane (Sigma, Saint Quentin Fallavier, France) was used as the standard, and thiobarbituric acid (TBA) as the reagent for MDA assay. 100 μL of either standard solutions or methanol extracts were injected in the HPLC system and the MDA–TBA adducts were detected.

2.3.2.4. Determination of SOD and POD enzyme activities. Total SOD activity was carried out in supernatants using the commercial kit (19160 SOD) (Sigma–Aldrich 3050 Spruce Street, St. Louis, MO 63103 USA). The activity of POD was determined according to the method described by Mitchell et al. (1994). A UV/Visible-spectrophotometer microplate reader (Multiskan GO, Thermo Scientific, France) was used to measure the SOD and POD enzyme activities.

2.3.2.5. Determination of protein content. Total protein content in supernatants was carried out using a UV/Visible-spectrophotometer microplate reader (Multiskan GO, Thermo Scientific, France). Protein content was measured by the bicinchoninic acid method using a commercial kit (BCA protein assay kit, Sigma–Aldrich, Saint-Quentin Fallavier, France) and BSA as standard (Smith et al., 1985).

2.3.2.6. Statistical analysis. Effects of diesel concentrations on the measured parameters were compared by ANOVA, using Statgraphics release 5.1 (Manugistic, Inc., Rockville, MD, USA). The means were obtained from five replicates. The method used to discriminate between the means was the LSD (Least Significant Difference 0.05) means comparison option. Diesel effects on spores' germination rate were evaluated with the Chi-square ($p < 0.05$) test available on the web site http://marne.u707.jussieu.fr/biostatgv/?module=tests/chideux ($p < 0.05$). The percentage data (mycorrhizal colonization, germination mode) were arcsine transformed.

3. Results

3.1. Diesel impact on the in vitro development of R. irregularis

3.1.1. Germination kinetics of R. irregularis spores in the absence and in the presence of diesel

Fig. 1 shows the kinetics of spore germination of *R. irregularis* cultivated *in vitro* after 3, 5, 7, 10, 20 and 30 days of incubation in the absence (control) and in the presence of increasing diesel concentrations (0.1, 0.25, 0.5 and 1%). Whereas, in the absence of diesel (control), the germination rate reached 92% after 3 days of incubation, it showed a significant reductions of 47, 53, 86 and 100% in the presence of 0.1, 0.25, 0.5 and 1% diesel concentrations, respectively.

3.1.2. Elongation of germinative hyphae of R. irregularis in the absence and in the presence of diesel

Hyphal lengths of germinative spore measured after 30 days of incubation in the absence (control) and in the presence of increasing diesel concentrations (0.1, 0.25, 0.5 and 1%) are shown in Fig. 2. Whereas the germinative hyphal lengths of *R. irregularis* spore were estimated about 2 cm after 30 days of incubation in the absence of diesel, a significant decrease of 87, 88, 92 and 100% was observed at 0.1, 0.25, 0.5 and 1% of diesel concentrations, respectively.

3.1.3. Germinative mode of R. irregularis spores in the absence and in the presence of diesel

In the absence of diesel (control), the majority of *R. irregularis* spores (92%) had a branched germinative mode (Photo 1a). Only 8% of spores had a linear germinative mode (Photo 1b) (Fig. 3). In the presence of diesel, the linear germinative mode has increased significantly at the expense of the branched germinative mode. At 0.1 and 0.25% diesel concentrations, branched germination rate was significantly reduced to 10 and 8% and linear germination rate reached respectively 90 and 92%. At 0.5% of diesel, all spores had a linear germinative mode (Fig. 3).

3.1.3.1. Spore viability test. All spores that had not germinated in the presence of the different concentrations of diesel, did not germinate after transfer in the diesel free medium.

3.2. Root colonization rate

Colonization of the chicory roots by the AM fungus *R. irregularis* after 9 weeks of incubation in the absence (control) and in the

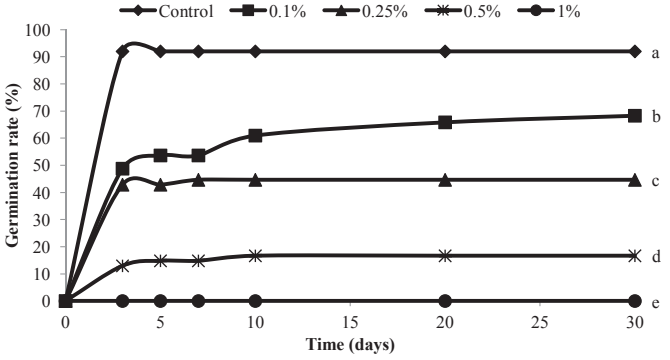

Fig. 1. *R. irregularis* spore germination kinetics during 30 days of incubation in the absence (control) and in the presence of increasing diesel concentrations (0.1, 0.25, 0.5 and 1%). The means were obtained from 50 replicates. Different letters indicate significant differences between spore germination kinetic plots according to the LSD test ($p < 0.05$).

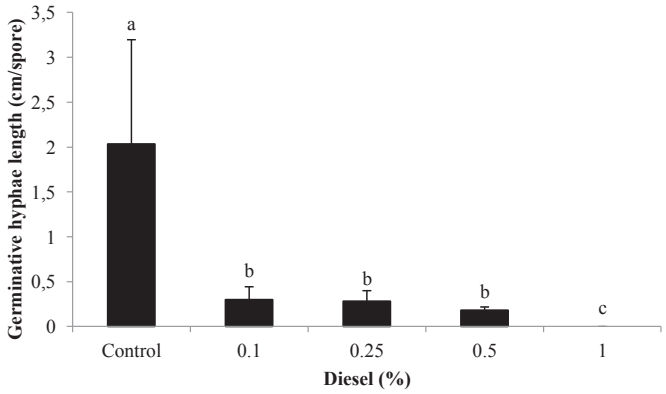

Fig. 2. *R. irregularis* germinative hyphae length after 30 days of incubation in the absence (control) and in the presence of increasing diesel concentrations (0.1, 0.25, 0.5 and 1%). The means were obtained from 50 replicates. Different letters indicate significant differences between spore germination kinetic plots according to the LSD test ($p < 0.05$).

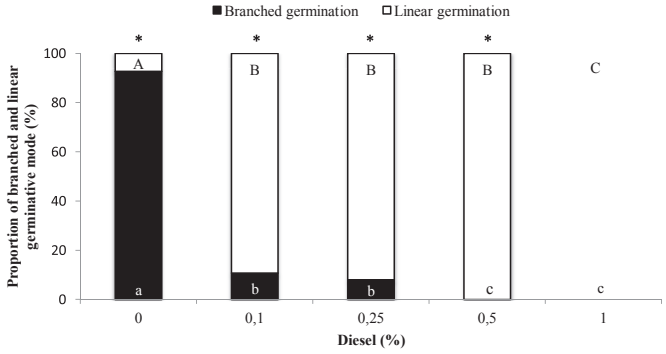

Fig. 3. Germinative modes of *R. irregularis* spores, observed after 30 days of incubation in the absence (control) and in the presence of increasing diesel concentrations (0.1, 0.25, 0.5 and 1%). The means were obtained from 50 replicates. Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$). *: Indicate a significant difference between branched and linear germination for each diesel concentration according to the LSD test ($p < 0.05$).

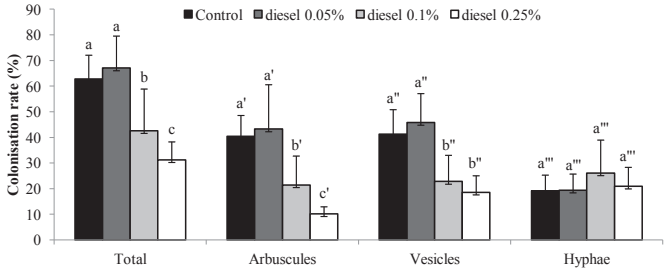

Fig. 4. Mycorrhizal colonization rates of chicory roots with the arbuscular mycorrhizal fungus *R. irregularis* grown in the absence (control) and in the presence of increasing diesel concentrations (0.05, 0.1 and 0.25%). The means were obtained from 5 replicates. Different letters indicate significant differences between the increasing diesel concentrations according to the LSD test ($p < 0.05$).

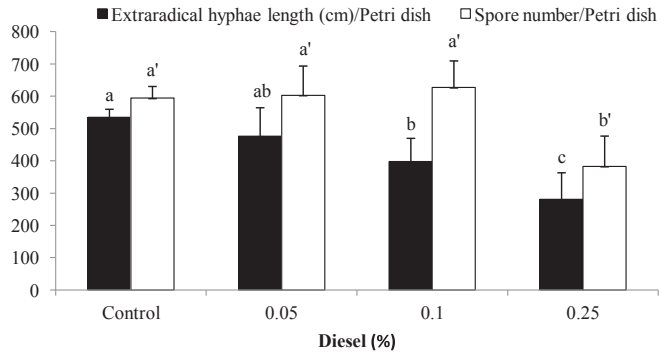

Fig. 5. Extraradical hyphae length and spore number of the AM fungus *R. irregularis* grown in the absence (control) and in the presence of increasing diesel concentrations (0.05, 0.1 and 0.25%). The means were obtained from 5 replicates. Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$).

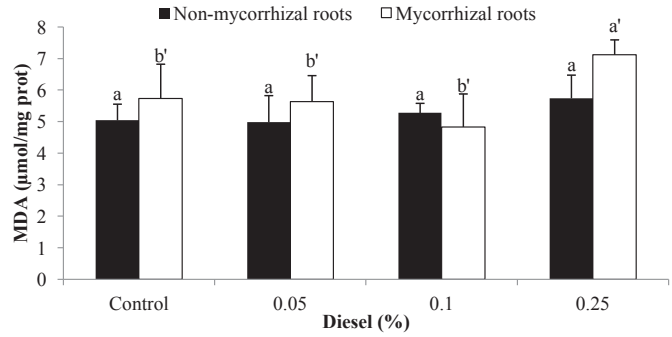

Fig. 6. Malondialdehyde (MDA) concentrations in non-mycorrhizal and mycorrhizal chicory roots grown during 9 weeks in the absence (control) and in the presence of increasing diesel concentrations (0.05, 0.1 and 0.25%). The means were obtained from 5 replicates. Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$).

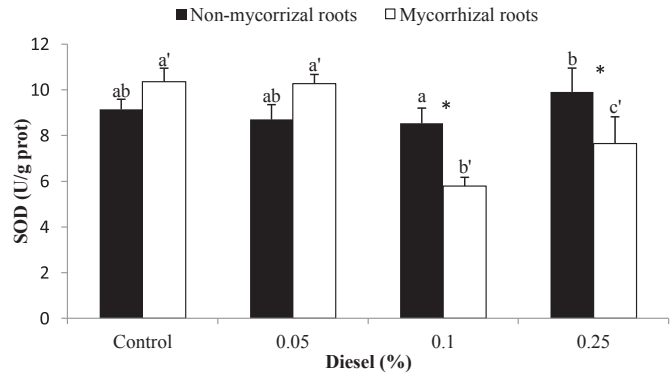

Fig. 7. Superoxide Dismutase (SOD) activity in non-mycorrhizal and mycorrhizal chicory roots grown during 9 weeks in the absence (control) and in the presence of increasing diesel concentrations (0.05, 0.1 and 0.25%). The means were obtained from 5 replicates. Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$). *: Indicate a significant different between non-mycorrhizal and mycorrhizal chicory roots for each diesel concentration according to the LSD test ($p < 0.05$).

presence of increasing diesel concentrations is shown in Fig. 4. Total mycorrhizal colonization rate was significantly ($p < 0.05$) decreased by 32 and 50% at 0.1 and 0.25% diesel concentrations. In the presence of the same diesel concentrations, arbuscules and vesicles have shown the same decline with significant decreases of 47 and 75% for arbuscules, and 45 and 55% for vesicles. In contrast, no significant difference was observed between the hyphal rates in the absence and in the presence of the various diesel concentrations ($P < 0.05$). The concentration 1% diesel has completely inhibited

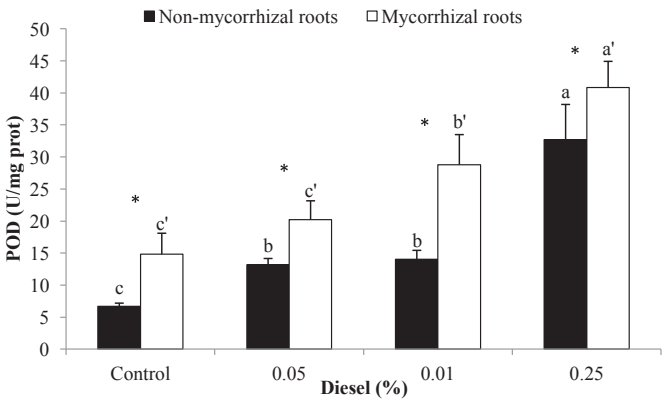

Fig. 8. Peroxidase (POD) activity in non-mycorrhizal and mycorrhizal chicory roots grown during 9 weeks in the absence (control) and in the presence of increasing diesel concentrations (0.05, 0.1 and 0.25%). The means were obtained from 5 replicates. Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$). *: Indicate a significant different between non-mycorrhizal and mycorrhizal chicory roots for each diesel concentration according to the LSD test ($p < 0.05$).

chicory roots growth.

3.3. *R. irregularis* extraradical development in the absence and in the presence of diesel

The average of extraradical hyphal lengths of the AM fungus *R. irregularis* grown in the absence of diesel (control) reached over 5.3 m. Whereas the concentration of 0.05% does not affect the extraradical hyphae development, the concentrations of 0.1 and 0.25% of diesel decrease it significantly when compared to the control ($p < 0.05$). These decreases were about 25 and 47%, respectively (Fig. 5).

The number of spores produced by the AM fungus *R. irregularis* remains constant in the absence (control) and in the presence of diesel concentrations 0.05 and 0.1%. In contrast, a significant decrease of 35% was observed at the diesel concentration of 0.25% (Fig. 5).

3.4. Diesel impact on the chicory roots growth

The effect of diesel on the growth of chicory roots colonized or not by the AM fungus *A. irregularis*, cultivated in the absence (control) and in the presence of diesel concentrations (0.05, 0.1, 0.25, 0.5 and 1%) was measured after 9 weeks of incubation. The average length of the non-mycorrhizal roots grown in the absence of diesel reached 8.1 m. At 0.25 and 0.5% diesel concentrations, reductions of 27 and 85% were observed. The average length of mycorrhizal roots reached 8.5 m in the absence of diesel. A significant decrease of 88% was observed in the presence of the concentration 0.5% of diesel (Table 1).

In the absence of diesel (control), the dry weights of mycorrhizal and non-mycorrhizal roots were 88.4 and 81.1 mg, respectively (Table 1). Whereas the dry weights of the mycorrhizal and non-mycorrhizal roots do not show any significant differences at the diesel concentrations 0.05 and 0.1%, they decreased significantly by 78 and 75% at the concentration of 0.5% when compared to the control. At the concentration 1%, root growth was completely inhibited (Table 1).

3.5. Diesel impact on oxidative stress parameters

3.5.1. Diesel impact on MDA content

The MDA amount produced by mycorrhizal and non mycorrhizal chicory roots in the presence of diesel remains constant when compared to the control. No significant difference was observed between the amount of MDA produced in mycorrhizal roots compared to non-mycorrhizal ones (Fig. 6).

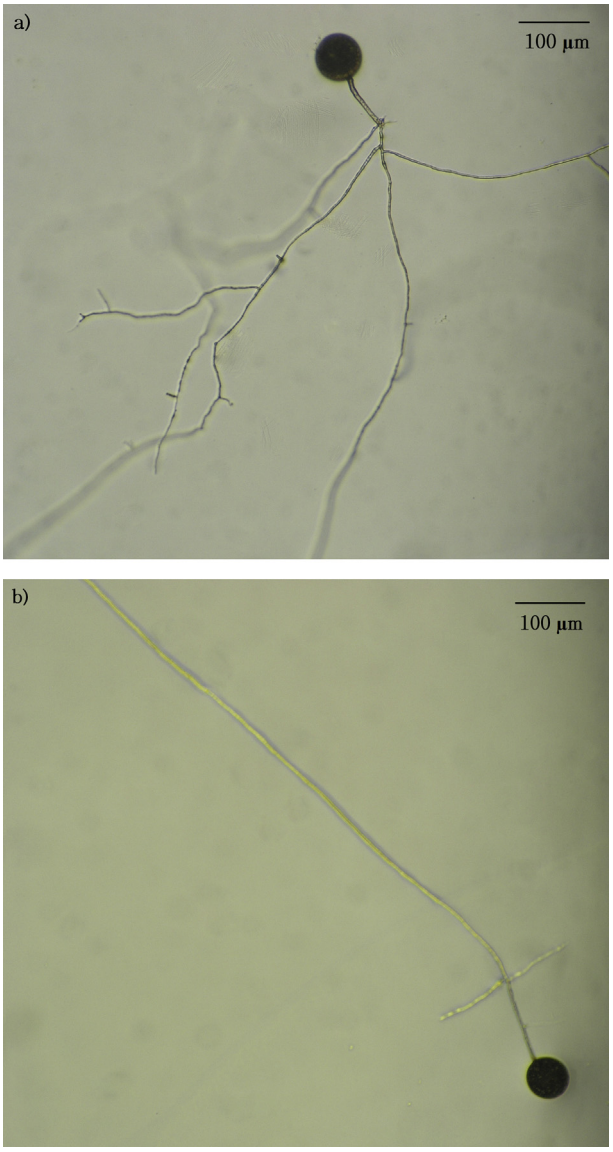

Photo 1. Germinative mode of *R. irregularis* spores: a) branched mode, b) linear mode, observed under an optical microscope (GX100).

3.5.2. Diesel impact on SOD activity

No significant change in SOD activity was observed in non-mycorrhizal roots in the absence and in the presence of diesel. However, a significant decrease in SOD activity was recorded in mycorrhizal roots at 0.1 and 0.25% diesel concentrations when compared to the control (Fig. 7). At 0.1 and 0.25% diesel concentrations, SOD activity was significantly lower in mycorrhizal roots compared to non-mycorrhizal ones.

3.5.3. Diesel impact on POD activity

Increasing concentrations of diesel has increased the POD activity in both mycorrhizal and non-mycorrhizal roots. The POD activity showed a significant increase in the mycorrhizal roots compared to non-mycorrhizal ones (Fig. 8). This increase was about

Table 1
Length and dry weight of non-mycorrhizal (NM) and mycorrhizal (M) chicory roots after 9 weeks of cultivation in the absence (control) and in the presence of different diesel concentrations (0.05, 0.1, 0.25, 0.5 and 1%). Different letters indicate significant differences between increasing diesel concentrations according to the LSD test ($p < 0.05$).

Diesel (%)	Root length (m/Petri dish)		Dry biomass (mg/Petri dish)	
	NM	M	NM	M
Control	8.1 ± 0.99^{a}	8.5 ± 0.94^{ab}	81.1 ± 4.69^{a}	88.4 ± 7.30^{a}
0.05	$7.6 \pm 0.62^{a*}$	8.9 ± 0.71^{a}	75.9 ± 3.61^{ab}	78.4 ± 5.51^{a}
0.1	$6.8 \pm 1.22^{b*}$	8.1 ± 0.55^{ab}	74.1 ± 12.58^{ab}	75.4 ± 6.55^{ab}
0.25	$5.9 \pm 1.85^{b*}$	7.7 ± 0.82^{b}	$38.5 \pm 10.09^{c*}$	62.4 ± 6.10^{bc}
0.5	1.1 ± 0.1^{c}	0.9 ± 0.1^{c}	20.4 ± 2.6^{d}	19 ± 3.2^{d}
1	0^{d}	0^{d}	0^{e}	0^{e}

*: Indicate a significant different between non-mycorrhizal and mycorrhizal chicory roots for each diesel concentration according to the LSD test ($p < 0.05$).

55, 35, 50 and 20% at 0, 0.05, 0.01 and 0.25% diesel concentrations respectively.

4. Discussion

Little is known about the effect of the hazardous fuel diesel on the AM fungi and the establishment of the plant beneficial AM symbiosis in the diesel contaminated soil. Thus, the present work consisted to study the impact of diesel on the main stages of the AM fungus *R. irregularis* development and on the root growth of the host plant, the chicory (*C. intibus* L.). Our results showed that the presence of diesel in the M medium at the concentrations of 0.1, 0.25, 0.5 and 1% not only causes significant decrease in the germination spore rates, with a complete inhibition at the highest concentration (1%), but also falls in the hyphal elongation as well as a change in the spore germinative mode. In fact, in the presence of the pollutant, spore germination in a linear mode occurs at the expense of spore germination in a branched manner. The linear germinative mode observed in the presence of diesel, could be a form of adaptation to the polluted medium in order to reduce the contact surface between the fungus and the pollutant. These results are in agreement with those found by Kirk et al. (2005) who report that 0.5% diesel concentration slowed down the germinative hyphae growth but did not affect the germination of *R. irregularis* spores cultivated *in vitro* and in the soil. Similar effects of other pollutant types (polycyclic aromatic hydrocarbons, fungicides) on *R. irregularis* spores have been reported (Alarcón et al., 2006; Debiane et al., 2008, 2009; Zocco et al., 2008; Calonne et al., 2010). Moreover, our findings showed that spores that did not germinate in the presence of diesel lost their germination ability denoting their mortality. These results suggest a fungicidal effect of diesel.

Addition of diesel into the culture medium has decreased significantly the formation of both arbuscules and vesicles rates in the AM fungus. Our results are in agreement with those of Tang et al. (2009) and Trejo et al. (2013) who have reported decreases in the mycorrhizal rates of *Zea mays* and *Brachiaria decumbens* cultivated in the presence of diesel. The decrease in the mycorrhizal colonization rate could be a consequence of both the reduction in the spore germination rate (Hirrel, 1981), and/or of the modification of the hyphal architecture. Diesel at 0.25% concentration had also negatively affected the *R. irregularis* extraradical development both in terms of hyphal elongation and spore formation. Similar effect on the extraradical development of *R. irregularis* was described by Campagnac et al. (2009) in the presence of fungicides. The presence of diesel had also a deleterious effect on the growth of the host roots. Both elongation and dry weight of mycorrhizal and non-mycorrhizal chicory roots have been decreased. It is noteworthy that after addition of diesel at the concentrations of 0.5 and 1%, the medium pH value decreased from 5.9 to 5.63 and 5.42 respectively. One can expect that these changes could be responsible for the observed root and fungal growth inhibition. However, several authors reported the ability of fungi and transformed culture to grow at a pH less than 5 (Mosse and Hepper, 1975; Green et al., 1992; Costa et al., 2013). This decline was more pronounced in the non-mycorrhizal roots as compared to the mycorrhizal ones. In fact, many studies with different mycorrhizal and non-mycorrhizal plants species (*Melilotus albus*, *Trifolium repens*, *Lolium perenne*, *Secale cereale*, *Sorghum bicolor*, *Z. mays*, *Onobrychis antasiacia*, *Pinus densiflora*, *Thuja orientalis* and *Populus tomentiglandulosa*) cultivated in the presence of diesel or diesel contaminated soil reported similar observation (Barrutia et al., 2011; Hernández-Ortega et al., 2012; Muratova et al., 2012; Jagtap et al., 2014). Our results show no increase in MDA, a biomarker of membrane lipid peroxidation, in the chicory roots in the presence of diesel, indicating that the diesel ecotoxicity cannot be explained by a membrane alteration. This decline in biomass could be the result of a deficiency in the water and mineral absorption (Quinones-Aguilar et al., 2003; Sangabriel et al., 2006). Hernández-Ortega et al. (2012) reported that plants of *M. albus* grown in diesel-contaminated soil had reduced content of some nutrient elements compared to plants grown in a non-contaminated. At 0.25% diesel concentration, mycorrhizal roots had greater dry weight than non-mycorrhizal ones thereby showing better growth when the fungus is present. These data suggest a protective effect of mycorrhization against the diesel toxicity. Such positive effect of mycorrhization was previously described in *B. decumbens* and *Lolium multiflorum* grown in a diesel contaminated soils (Alarcón et al., 2008; Trejo et al., 2013). The protective effect of mycorrhization has been also observed in the presence of other pollutants (polycyclic aromatic hydrocarbons, heavy metals) (Joner and Leyval, 2003; Debiane et al., 2009; Redon et al., 2009).

According to our study, it seems that one way by which AM inoculation protects chicory roots could be the induction of the POD antioxidant enzyme activity. Indeed, increased diesel concentrations resulted in a higher increase of POD activity in the mycorrhizal roots. The induction of POD activity would contribute to eliminate more effectively the ROS accumulated under diesel stress exposure. These results are in agreement with those of Campagnac et al. (2010) who reported an increase in POD activity in mycorrhizal roots in the presence of the fungicide fenpropimorph. The same effect was observed by Tang et al. (2009) on the *Z. mays* mycorrhizal plants grown in the presence of diesel.

However, the SOD activity remained constant in the non-mycorrhizal roots and decreased in the mycorrhizal ones in the presence of the diesel concentrations 0.1 and 0.25%. These results are contradictory with other studies that report an increase in SOD activity in mycorrhizal roots (Debiane et al., 2009; Tang et al., 2009). The decrease in SOD activity could be due to (i) a low generation of $O_2^{\cdot-}$ in mycorrhizal roots, (ii) the involvement of other enzymatic or non-enzymatic antioxidant systems such as vitamin C (ascorbate), molecules involved in the trapping of $O_2^{\cdot-}$, (iii) the inactivation of the SOD enzyme by the ROS. At 0.25% diesel concentration, an improvement of mycorrhizal root growth accompanied with a decrease of SOD activity was observed. This could be a result from the attenuation of oxidative stress related to the presence of the AM fungus. To validate this hypothesis, it would be interesting to study, in future, expression of the genes involving in the antioxidant systems implemented by the AM fungus to protect plants against the diesel phytotoxicity.

5. Conclusion

Taken together, our findings show that diesel pollution affects negatively the main stages of the AM fungus *R. irregularis* development and the growth of the chicory roots, which were not completely inhibited except at the highest tested concentration (1%). The mycorrhizal fungus was not only able to fulfil its life cycle in the presence of diesel but it was also able to provide protection to chicory root against the diesel toxicity. The protective effect could result from the induction of the antioxidant POD enzyme but not to SOD enzyme. Furthermore, alteration of membrane lipids does not appear to be responsible of the observed phytotoxicity. Generally, this work not only contributes to the assessment of the toxicity of pollutants from industrial sources on the AM symbiosis, but also allows to provide a tool in the soil ecotoxicology studies and risk assessment. In addition, this work highlights the importance of *in vitro* cultures to investigate the mechanisms of pollutant toxicity on the beneficial flora in the soil such as AM fungi.

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