DIESEL FUEL DIFFERENTIALLY AFFECTS HYPHAL HEALING IN GIGASPORA SP. AND RHIZOPHAGUS IRREGULARIS

Monica Cristina Garces Ruiz, Maryline Calonne-Salmon, Vincent Bremhorst, Stephan Declerck

REPRINT | 2021 / 02

UCLouvain

SMCS | LIDAM Louvain Institute of Data Analysis and Modeling in economics and statistics

SMCS

Voie du Roman Pays 20, L1.04.01 B-1348 Louvain-la-Neuve

Tel (32 10) 47 94 07

Email: smcs-stat@uclouvain.be

https://sites.uclouvain.be/training/smcs/

Mycorrhiza (2021) 31:413–421
https://doi.org/10.1007/s00572-021-01026-5

SHORT NOTE

Diesel fuel differentially affects hyphal healing in *Gigaspora* sp. and *Rhizophagus irregularis*

Mónica Garcés-Ruiz[1] · Maryline Calonne-Salmon[1] · Vincent Bremhorst[2] · Stéphane Declerck[1]

Received: 16 November 2020 / Accepted: 22 February 2021 / Published online: 4 March 2021

Abstract

Hydrocarbon pollution is an increasing problem affecting soil ecosystems. However, some microorganisms can cope with these pollutants and even facilitate plant establishment and thus phytoremediation. Within soil, arbuscular mycorrhizal fungi (AMF) have developed several strategies to survive and flourish under adverse conditions. Among these is the hyphal healing mechanism (HHM), a process allowing hyphae to re-establish integrity after physical injury. This mechanism differs among species and genera of AMF. However, whether and to what extent hydrocarbon pollution impacts the HHM is unknown. Here, the HHM was monitored in vitro on two AMF strains, *Rhizophagus irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331, under increasing concentrations of diesel (1, 2, and 5% v:v). The addition of diesel slowed-down the HHM in both fungi. On *Gigaspora* sp., this effect was limited and most hyphae were able to heal after injury. Conversely, all steps of healing were severely impaired in *R. irregularis*. That fungus reconnected the injured hyphae at a much lower frequency than the *Gigaspora* sp., instead investing its energy to link neighboring hyphae or roots, or developing new branches from uninjured hyphae.

Keywords Arbuscular mycorrhizal fungi · *Cichorium intybus* L. · Diesel fuel · Hyphal healing mechanism · Common mycorrhizal network · Hydrocarbon pollutant

Introduction

Crude oil pollution from exploration and processing of petroleum is widespread in water and/or soil (Vwioko and Omamogho 2012). Oil pollution influences soil microbial communities (Amadi et al. 1996; Nicolotti and Egli 1998; Gan et al. 2009; Abha and Singh 2012; Joye et al. 2014) with putative effects on ecosystem functioning, soil fertility, and environmental quality (Peng et al. 2015). Diesel fuel in worldwide use by machinery such as power generators and vehicles is a distilled mixture from crude oil containing a combination of 65–85% saturates, 5–30% aromatics, and 0–5% olefins. Nevertheless, these percentages are variable according to the petroleum source, the refining process, and the sulfur content (Liang et al. 2005). Diesel is toxic to the environment, owing to the presence of low molecular weight compounds with high volatility, solubility, and bioavailability, such as polyaromatic hydrocarbons (PAHs) and alkanes (Dorn and Salanitro 2000). Its accumulation in soil is particularly harmful to plants and their microbial root-associates as well as a source of contamination of ground water (Adam and Duncan 1999; Driai et al. 2015). However, the ability of microbial communities to degrade hydrocarbon compounds has been reported in several studies (Kauppi et al. 2011; Faure et al. 2015; Stefani et al. 2015), providing information for bioremediation of petroleum-contaminated sites. Microorganism-assisted phytoremediation has been reported to enhance hydrocarbon degradation (Joner and Leyval 2001; Merkl et al. 2005; Gan et al. 2009).

Arbuscular mycorrhizal fungi (AMF) are key players in the rhizosphere. Besides promoting plant growth, AMF sustain other ecological and economically important functions such as improved plant resistance/tolerance to salinity and drought (Plouznikoff et al. 2016) and also to organic pollutants such as PAHs (Leyval and Binet 1998; Verdin et al. 2006; Debiane et al. 2008, 2009; Lenoir et al. 2017; Calonne-Salmon et al. 2018), diesel (Kirk et al. 2005;

✉ Stéphane Declerck
stephan.declerck@uclouvain.be

[1] Laboratory of Mycology, Earth and Life Institute, Université catholique de Louvain, Croix du Sud 3, 1348 Louvain-la-Neuve, Belgium

[2] Statistical Methodology and Computing Service, Université catholique de Louvain, Voie du Roman Pays 20, 1348 Louvain-la-Neuve, Belgium

Tang et al. 2009), and crude oil (Cabello 1999; Salami and Elum 2010; Nwoko 2014). These microorganisms are thus considered potential agents for remediation strategies (Lenoir et al. 2016; Rajtor and Piotrowska-Seget 2016). AMF can facilitate plant establishment and survival in hydrocarbon-contaminated soils by stimulating the activity of soil microbial enzymes which contribute to the biodegradation of hydrocarbons in the rhizosphere (Meglouli et al. 2019). Conversely, a number of studies have reported the impact of oil pollutants (e.g., benzo[a] pyrene, anthracene, phenanthrene, and diesel) on AMF development and physiology (Kirk et al. 2005; Franco-Ramírez et al. 2007; Debiane et al. 2008, 2009; Calonne et al. 2014a; Driai et al. 2015). These authors generally observed a significant decrease in spore germination, hyphal length, root colonization, and P transport of different species of the family Glomeraceae in the presence of hydrocarbons. Thus, AMF may also be considered as bio-indicators of soil pollutants (ISO 2009; de Novais et al. 2019).

A fascinating feature of AMF is the ability of their extraradical mycelium to develop through the soil and interconnect individual plants of the same or different species to form "common mycorrhizal networks" (Simard et al. 2012). These "mycorrhizal webs" are thought to play key roles in ecosystems (Giovannetti et al. 2015). These networks also may result from the interconnection of hyphae via a mechanism called anastomosis (Giovannetti et al. 1999, 2001, 2004; Kirk et al. 2001; de Souza and Declerck 2003; de la Providencia et al. 2005; Voets et al. 2006, 2009; Purin and Morton 2011). Anastomosis formation concerns intact hyphae, and it has been linked to damaged hyphae as well, the latter being described as a hyphal healing mechanism (HHM) (de la Providencia et al. 2005). The HHM is a major process allowing the fungi to maintain mycelial integrity following physical injury. In some Gigasporaceae (e.g., *Scutellospora reticulata*, *Gigaspora margarita*, *Gigaspora rosea*), reconnection of cut hyphae is the main mechanism of repair (de Souza and Declerck 2003; de la Providencia et al. 2005). Conversely, in some Glomeraceae (e.g., *Rhizophagus. irregularis*, *R. proliferus*, and *R. clarus*, previously known as *Glomus intraradices*, *Glomus proliferum*, and *Glomus clarum*, respectively, and *Glomus hoi*), high numbers of new hyphae are produced at both sides of hypha injuries. Those new hyphae are capable of reconnecting severed hyphae but also may anastomose with uninjured hyphae or may link to neighboring roots (de la Providencia et al. 2005, 2007).

The HHM has been investigated in vitro on species belonging to the Glomeraceae and Gigasporaceae (de la Providencia et al. 2005, 2007). Four events from physical injury to complete fusion with re-establishment of cytoplasmic/protoplasmic flow have been described for the HHM: (a) septum formation; (b) initiation of growing hyphal tips (GHTs developed at one or both sides of the cut hyphae); (c) GHTs elongation, orientation, and contact; and (d) GHTs fusion and re-establishment of cytoplasmic/protoplasmic flow (de la Providencia et al. 2005). These four events differentiated the Glomeraceae and Gigasporaceae (de la Providencia et al. 2005). However, environmental factors may influence extraradical mycelium functioning. For instance, it is unknown whether and to what extent hydrocarbon pollutants such as diesel may affect the HHM of members belonging to the two mentioned families of AMF, and thus may affect their integrity and functions. From an applied perspective, this may have important implications for the use of AMF inoculants in phytoremediation of hydrocarbon-polluted soils.

In the present study, *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331 were grown in vitro on transformed root organ cultures (ROC) (IJdo et al. 2011) of chicory. We tested the hypothesis that increasing (1, 2 and 5% v:v) concentrations of diesel (a hydrocarbon derivative) affect the HHM of both fungi differently. Diesel was applied on injured hyphae, and the healing mechanism was monitored and analyzed by microscopy.

Materials and methods

Biological material

The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker, and Buscot) C. Walker and A. Schüßler as ("irregulare") MUCL 41833 and *Gigaspora* sp. (Gerdemann and Trappe) MUCL 52331 were supplied by the Glomeromycota in vitro collection (GINCO—http://www.mycorrhiza.be/ginco-bel) and maintained on Ri T-DNA transformed chicory (*Cichorium intybus* L., clone A4NH) roots (Fontaine et al. 2004) as described by Cranenbrouck et al. (2005).

Preparation of MSR-diesel medium

Four 100-mL bottles (VWR, USA) containing 50 mL of modified Strullu–Romand (MSR) medium (Declerck et al. 1998) supplemented with 3 g L^{-1} GelRite® (Carl Roth, Germany) and a stirring magnet were sterilized (121°C/1 bar for 15 min). Filtered (0.45 µm Millipore-Merck, USA) winter diesel blend from a gas station (Q8, Louvain la Neuve, Belgium) was added to the bottles at 1, 2, or 5% (v:v of MSR medium) concentrations and maintained in agitation at 40°C before use. One bottle without diesel was used as the Control.

Diesel concentrations were selected according to a preliminary experiment with *Gigaspora* sp. and 2% diesel. The concentration of 2% showed a slowdown in the healing mechanism; however, re-establishment of cytoplasmic/

protoplasmic flow was observed in several cut hyphae (data not shown).

Experimental design

The two AMF strains were grown in Petri dishes (135-mm diam.) in association with transformed chicory roots. The Petri dishes were filled with 90 mL of MSR medium as follows. Briefly, the Petri dishes were inclined at 5° during filling with the MSR medium to ensure a slight slope from one to the other end of the Petri dishes during solidification of the medium. The AMF were associated with the chicory roots in the thickest part of the MSR medium. Roots tended to grow preferentially in this part, while the hyphae tended to develop more easily in the thin part of the MSR medium facilitating microscopic observations. The Petri dishes were incubated in an inverted position at 27°C in the dark.

After 1 month of incubation, Petri dishes of each fungus showing optimal production of mycelium were selected. In each of them, between 6 and 14 runner hyphae growing close to the surface of the medium and showing dense cytoplasmic/protoplasmic flow under a dissecting microscope (with bright-field light illumination Olympus BH2–RFCA, Japan), at × 200 magnification, were carefully chosen and numbered without opening the dishes. The selected hyphae were chosen at a reasonable distance (~5–10 mm) from each other to avoid close contacts or direct interconnectedness. The hyphae were cut with a sterile scalpel under a binocular microscope (Olympus SZ2-ILST, Japan) at × 6.7 or × 40 magnification. Immediately after cutting, 20 µL of MSR-diesel medium containing 1, 2, or 5% concentration or MSR medium without diesel (i.e., the Control), maintained in agitation at 40°C was added with a micropipette at the cutting sites. Performing the cuts and adding the MSR medium supplemented or not with diesel in one Petri dish were done at the same time. Each cut plus addition of the medium in a single dish took approximately 1 min, and the times of cutting were tracked precisely.

Monitoring of the hyphal healing mechanism

The cut hyphae were inspected cautiously at regular intervals under the dissecting microscope at × 100 or × 200 magnification. Immediately after the cutting, and during the first 60 min, the hyphae in each Petri dish were observed. In some hyphae the first steps of healing already were observed at that time (i.e., the formation of a plug of cytoplasm/protoplasm, change of color around the injured hyphae, septum formation). Then each cut was evaluated under the microscope every hour for 16 h and finally at 24, 30, 36, and 48 h. After hyphal cutting, every change was noted to be compared at the end of the experiment. The HHM was analyzed in injured hyphae belonging to three AMF colonies (i.e., three Petri dishes) per strain (*Gigaspora* sp. and *R. irregularis*) and for each treatment (i.e., Control and 1, 2, 5% diesel), yielding between 19 and 40 cut hyphae observed per treatment (see Table 1).

Images of the GHTs were captured with a digital camera (Canon EOS 60D) coupled to a compound bright-field light microscope to reconstitute the sequence of HHM using image manager software (EOS Utility Canon USA Inc.).

Data collection

Following cutting, the time to septum formation, GHTs initiation at one or both sides of the cut hyphae, contact and fusion between GHTs, and subsequent cytoplasmic/protoplasmic flow were recorded on each cut hypha. In addition, the number of new GHTs from cut hyphae as well as the number of ramifications (i.e., branches that were produced from the GHTs) was recorded. The number of cut hyphae that became empty and showed multiple retraction septa also was recorded.

Statistical analysis

Our response variables are defined as the time necessary to observe septum formation, GHTs initiation at one or both sides of the cut hyphae, contact and fusion between GHTs, and subsequent cytoplasmic/protoplasmic flow. Such outcomes need to be analyzed within a time-to-event modeling framework, known as survival analysis. The most used survival or time-to-event model is the Cox model (Cox 1972; George et al. 2014).

In this study, an extension of the Cox model that enabled us to deal with interval censored data has been used (cf. monitoring section describing the post cutting evaluation schedule). Precisely, each event-time was analyzed independently, using the *MIICD.coxph* function of the MIICD R package (Delord 2015). Non-observed events after 48 h were considered as right censored. The percentage of observed events within each treatment group and the results obtained from the Cox model are reported in Table 1.

Interpretation of the Cox model

A negative coefficient estimate in a Cox model indicates that the hazard ratio between a given treatment (1, 2, or 5% diesel) and the Control (without diesel) is smaller than one, meaning that the hazard function of the treatment is smaller than the hazard function of the Control. In other words, in the given treatment, the event occurs later or is less frequently observed than in the Control. The interpretation of a positive estimate in the Cox model implies a hazard ratio larger than one, meaning that the event occurs sooner or

Table 1 Number of cut hyphae and descriptive percentage of observed events of the hyphal healing mechanism of *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833 in the absence (Control) or presence of diesel 1% (Diesel 1%), 2% (Diesel 2%), or 5% (Diesel 5%) added at the cutting site, the last observation after 48 h. The hazard ratios obtained from the Cox model (see "Statistical analysis" subsection) and their significance (*p*-values) are reported for each treatment. Significant probabilities are shown in italics

Observed Event	*Gigaspora* sp. MUCL 52331					*R. irregularis* MUCL 41833			
	Number of cut hyphae, %	% of hyphae showing	Hazard ratio	*p*-value	Treatment	Number of cut hyphae, %	% of hyphae showing	Hazard ratio	*p*-value
Septum formation at both sides of cut ends	21 (25.3)	95	-	-	Control	40 (34.2)	70	-	-
	22 (26.5)	86	0.353	< 0.001	1%	28 (23.9)	32	0.365	0.009
	21 (25.3)	76	0.285	< 0.001	2%	20 (17.1)	35	0.403	0.032
	19 (22.9)	68	0.175	< 0.001	5%	29 (24.8)	38	0.434	0.020
First GHTs emergence	21 (25.3)	90	-	-	Control	40 (34.2)	100	-	-
	22 (26.5)	86	0.477	0.024	1%	28 (23.9)	71	0.125	< 0.001
	21 (25.3)	86	0.488	0.031	2%	20 (17.1)	55	0.071	< 0.001
	19 (22.9)	79	0.293	< 0.001	5%	29 (24.8)	79	0.156	< 0.001
GHTs emergence at both sides of cut ends	21 (25.3)	90	-	-	Control	40 (34.2)	80	-	-
	22 (26.5)	82	0.528	*0.054*	1%	28 (23.9)	32	0.172	< 0.001
	21 (25.3)	71	0.397	< 0.001	2%	20 (17.1)	40	0.208	< 0.001
	19 (22.9)	52	0.217	< 0.001	5%	29 (24.8)	48	0.298	< 0.001
GHTs contact	21 (25.3)	86	-	-	Control	40 (34.2)	45	-	-
	22 (26.5)	64	0.370	0.005	1%	28 (23.9)	18	0.292	0.015
	21 (25.3)	81	0.452	0.020	2%	20 (17.1)	5	0.079	0.013
	19 (22.9)	47	0.207	< 0.001	5%	29 (24.8)	10	0.167	0.004
Re-establishment of cytoplasmic/ protoplasmic flow	21 (25.3)	86	-	-	Control	40 (34.2)	7.5	-	-
	22 (26.5)	59	0.419	0.017	1%	28 (23.9)	7	0.602	0.651
	21 (25.3)	57	0.354	0.005	2%	20 (17.1)	5	0.638	0.697
	19 (22.9)	37	0.194	< 0.001	5%	29 (24.8)	3	0.438	0.475
Retraction	21 (25.3)	14	ND		Control	40 (34.2)	78	ND	
	22 (26.5)	23			1%	28 (23.9)	32		
	21 (25.3)	29			2%	20 (17.1)	30		
	19 (22.9)	16			5%	29 (24.8)	21		

ND not determined

Significant values reflect $p < 0.05$ compared with the Control treatment (Cox model test)

is more frequently observed in the given treatment compared with the Control.

Results

Hyphal healing mechanism in *Gigaspora* sp. MUCL 52331 in the presence of increasing concentrations of diesel

Whatever the treatment, cytoplasmic/protoplasmic loss was noticed at both sides of the cut hyphae with the formation of a plug at one or both cut ends (Fig. 1a). This was followed by the rapid formation of a septum in more than 68% of the hyphae whatever the concentration of diesel, while in the Control treatment, it reached 95% (Table 1). The section distal to the septum changed color and turned darker (Fig. 1a). The impact of diesel on septa formation at both cut ends was significant. The hazard ratios comparing the 1, 2, and 5% diesel treatments with the Control treatment were 0.353, 0.285, and 0.175 ($p < 0.001$), respectively (Table 1), meaning that the event was observed sooner and more frequently in the Control treatment as compared with the diesel treatments.

The emergence of GHTs was always initiated behind the septum (Fig. 1a) or distant from the cut end when no septum was visible. Whatever the treatment, the percentage of hyphae showing at least one GHT emergence exceeded 79% (Table 1). Following the first GHT emergence, a second GHT was generally formed in the hyphae on the other side of the cut. The percentage of observed GHT formation at both cut ends differed among treatments. In the 5% diesel treatment, GHTs formation at both ends occurred only in

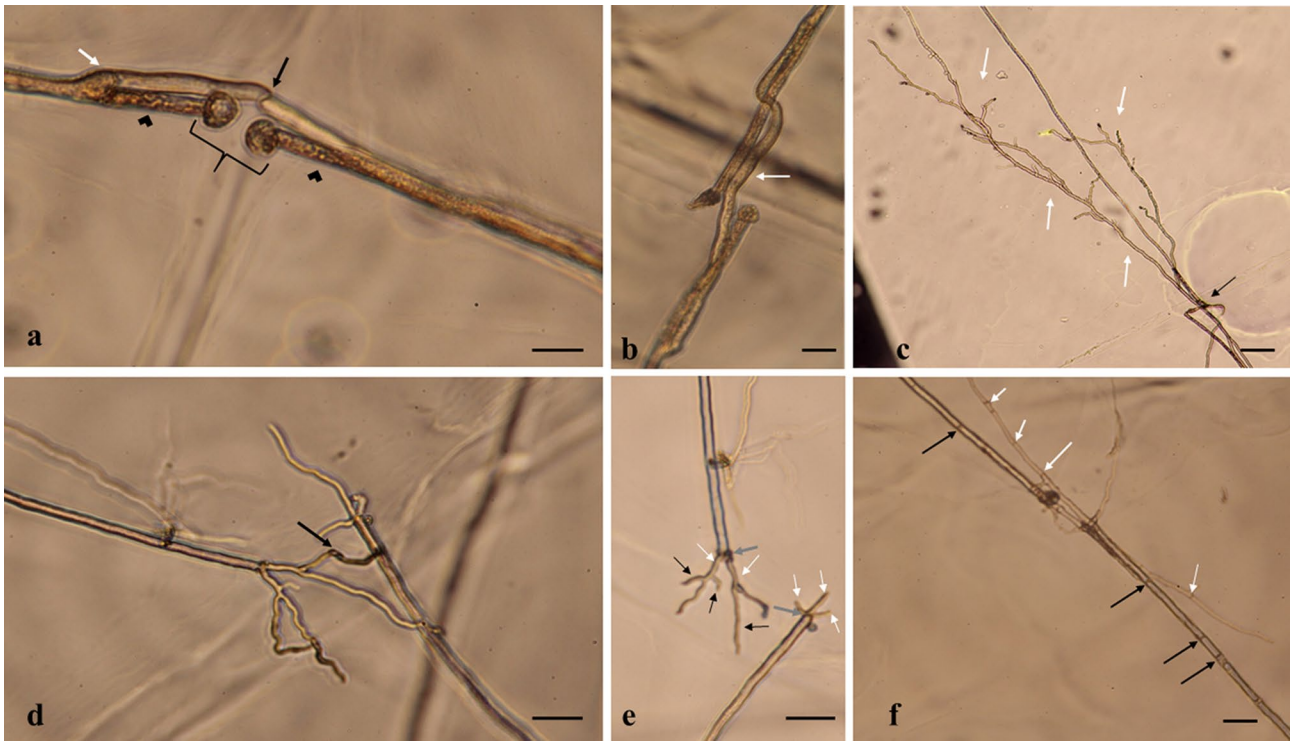

Fig. 1 Impact of diesel on the hyphal healing mechanism in *Gigaspora* sp. MUCL 52331 and *Rhizophagus irregularis* MUCL 41833. From a–c *Gigaspora* sp. **a** At 5% diesel concentration. Plugs were formed at both cut ends (brace). The cut ends behind the plug changed color turning darker (arrowheads). Growing hyphal tips (GHTs) emerged at both ends (white arrow) and contacted each other (black arrow) (scale bar 50 µm). **b** At 1% diesel concentration contact and fusion of emerging hyphae (white arrow) (scale bar 50 µm). **c** Control treatment. Development of ramifications (white arrows) from one side of cut hypha (black arrow, scale bar 100 µm) at the end of the experiment. From **d–f** *Rhizophagus irregularis*. **d** Control treatment. GHTs contact and fusion following cutting (black arrow) (scale bar 50 µm). **e** Control treatment. Septa formation after cutting with no plug formation (grey arrows). GHTs emerging (white arrows) through the cut ends and ramification developed (black arrows) from GHTs (scale bar 50 µm). **f** Control treatment showing septa formation in the hypha (black arrows) and in GHTs (white arrows) (scale bar 50 µm)

52% of the cases, while this percentage increased to 71% and 82% for the 2% and 1% diesel treatments, respectively, and reached 90% in the Control treatment. The Cox model showed a significant impact of diesel on GHTs formation. This formation (first emergence (Supplementary Figure S1) or at both ends) occurred significantly later or less often in the diesel treatments compared with the Control treatment (the hazard ratios were significantly lower than one). However, the response to the 1% diesel did not differ from the Control treatment for the GHT emergence at both sides of cut ends event ($p = 0.054$; Table 1).

Similar results were obtained for the GHTs contacts and for the re-establishment of cytoplasmic/protoplasmic flow (Fig. 1b). The presence of diesel reduced (or had them occur later) the healing compared with the Control treatment (Fig. S1; Table 1).

Arrest of cytoplasmic/protoplasmic flow was observed a few times following complete healing. The percentage of cytoplasmic retraction (i.e., septa formation) of cut hyphae, ramifications, or GHTs (Fig. 1c) was recorded after 48 h. A higher percentage of cytoplasmic retraction was observed in the 1% and 2% diesel treatments, compared with Control and 5% diesel treatments (i.e., descriptive statistics; Table 1).

Hyphal healing mechanism in *R. irregularis* MUCL 41833 in the presence of increasing concentrations of diesel

Whatever the treatment, cytoplasmic/protoplasmic loss was nearly always noticed at both sides of the cut hyphae. The formation of a plug was rarely observed (Fig. 1e). The percentage of septum formation in the cut hyphae was above 70% for the Control treatment, but below 38% in the diesel treatments (Table 1).

As revealed by the results of Cox model analyses (Table 1), diesel had a significant impact on septum formation. It occurred less frequently or later in the diesel treatments compared with the Control treatment.

Similar results were obtained for the GHTs formation (first emergence (Fig. S1) and at both cut ends) and for the GHTs contact events (Table 1), while no effect of diesel was reported on the re-establishment of cytoplasmic/

protoplasmic flow compared with the Control (Fig. 1d; S1; Table 1). Non-fused GHTs showed retraction with septa formation (Fig. 1f). The percentage of hyphae showing retraction was higher in the Control treatment (78%) compared with the diesel treatments (less than 32%) (Table 1).

Comparison of the hyphal healing mechanism between *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833 in the presence of increasing concentrations of diesel

Gigaspora sp. and *Rhizophagus irregularis* differed in their response to injury as well as to diesel application. For both fungi, the complete healing with re-establishment of cytoplasmic/protoplasmic flow was strongly delayed or less frequently observed in the presence of diesel, notably at the highest concentration (i.e., 5%).

For both AMF, septum formation was delayed or less frequently observed in the presence of diesel, whatever its concentration. Following septum formation, GHTs emerged at one or both cut ends of the injured hyphae. In the presence of diesel, the capacity to produce one GHT at one of the cut ends was slightly affected in *Gigaspora* sp.: GHTs formation dropped by 12% in the treatment with 5% diesel and by ~ 4% in the treatments with 1% and 2% diesel compared with the Control treatment (90%). Conversely, in *R. irregularis* it was drastically affected. GHTs formation, at least at one cut end, dropped by ~29, 45 and 21% compared with the Control in the presence of 1, 2, and 5% diesel, respectively. The production of at least one GHT at both sides of the cut end was affected as well. In *Gigaspora* sp., it was decreased by 42% at the highest diesel concentration compared with the Control treatment, while for *R. irregularis* it was decreased by ~63, 50, and 40% compared with the Control treatment in the presence of 1, 2, and 5% diesel, respectively.

The majority of GHTs at both ends of the cut hyphae in *Gigaspora* sp. grew toward each other and in most cases (i.e., 86, 64, 81, and 47% for the Control, 1, 2, and 5% diesel, respectively (Table 1)) entered into contact. To the contrary, only 45% (Table 1) of the GHTs in *R. irregularis* entered into contact in the absence of diesel and this percentage dropped by ~60, 89, and 78% compared with the Control in the presence of 1, 2, and 5% diesel. Contact of GHTs was followed by fusion and re-establishment of cytoplasmic/protoplasmic flow in *Gigaspora* sp.; however, this event was delayed at increasing concentrations of diesel compared with the Control treatment. In *R. irregularis*, GHT contacts were also observed, which in 7.5% of the cases resulted in re-establishment of cytoplasmic/protoplasmic flow in the absence of diesel. In the presence of 1, 2, and 5% diesel, 7, 5, and 3% of the cases, respectively, resulted in re-establishment of cytoplasmic/protoplasmic flow (Table 1).

Discussion

The impact of anthropogenic pollution, such as diesel spillage, was studied on the HHM mechanism of *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833, two AMF belonging to phylogenetically distant families, under in vitro culture conditions.

For both AMF, septum formation was observed a few minutes after injury, at the cut ends for *R. irregularis* and some micrometers behind the cut ends for *Gigaspora* sp. as earlier observed by de la Providencia et al. (2005). Moreover, GHTs emerged at one or both cut ends of the injured hypha, behind the septum for *Gigaspora* sp. and most often through the septum or lumen at the site of the cut end for *R. irregularis* as previously observed by de la Providencia et al. (2005). Nevertheless, for both AMF strains, the events of healing were delayed or less frequently observed in the presence of diesel, whatever its concentration.

The GHTs emergence in *Gigaspora* sp. seemed to be impaired by increasing concentrations of diesel. To the contrary, the GHTs emergence in *R. irregularis* dropped severely in the diesel treatments and also dropped in the Control. The decrease observed for *Gigaspora* sp. at the highest concentration, and at all concentrations for *R. irregularis*, may be related to the impact of hydrocarbons on membrane composition, thus inhibiting signal recognition. Indeed, persistent organic pollutants such as benzo[a]pyrene and anthracene have been demonstrated to induce a slowdown in the sterol biosynthesis pathway (Calonne et al. 2014a, b), leading to a decrease of 24-methylcholesterols in membranes (Debiane et al. 2011). It is well known that sterols contribute to the normal functioning of plasma membranes (fluidity regulation and permeability) which are correlated with the mycelial growth and physiology of fungi (Weete 1989; Vanden Bossche 1990; Dalpé et al. 2012). A reduction in the capacity of the fungus to recognize signal and for GHTs to grow toward each other could thus be strongly affected by diesel. Similarly, phosphatidylcholine (the main phospholipid in AMF) content has been demonstrated to decrease in extraradical mycelium in the presence of PAHs and was linked with the induction of oxidative stress in the extraradical network (Debiane et al. 2011; Calonne et al. 2014b). Perturbation of membrane composition and content could thus be inferred for the impact of diesel on signal recognition. The impact of diesel has been studied in the life cycle of *R. irregularis* and its host transformed chicory roots (Driai et al. 2015). Those authors demonstrated that mycorrhizal or non-mycorrhizal root growth was reduced by 88 and 85%, respectively, in the presence of 0.5% diesel. Moreover, at

1% diesel concentration the growth of transformed roots was completely inhibited. The different concentrations of diesel (0.1, 0.25, 0.5, and 1%) used during their study demonstrated a decrease in spore germination rates and hyphal elongation, showing a complete inhibition at the highest concentration (Driai et al. 2015).

Contact of GHTs was always followed by GHT/GHT or GHT/PEG (a term used to identify a very short specialized branch (de la Providencia et al. 2005)) fusion with re-establishment of cytoplasmic/protoplasmic flow in *Gigaspora* sp. The GHT/PEG fusion was observed for a few cut hyphae, where only one GHT was present at one side of the cut, but nevertheless, the re-establishment of cytoplasmic/protoplasmic flow was observed. As for the other events, contact was delayed at increasing concentrations of diesel compared with the Control treatment. In *R. irregularis*, GHT contacts were also observed, with a low re-establishment of cytoplasmic/protoplasmic flow in the absence of diesel as well as in its presence. With this fungus, a pre-contact interaction between the GHTs, especially in the Control treatment (45%) previously had been observed, probably via a remote signaling process (de Novais et al. 2017), but ended by overlapped GHTs without further fusion. This interaction has been observed and classified by Sbrana et al. (2011) as hyphal close contact with no fusion and no morphological changes.

Interestingly, in *Gigaspora* sp., the ramifications of fused GHTs as well as of GHTs that did not fuse, stopped growth, and septa formations were observed. This suggests a very specific role of GHTs to fuse with neighboring GHTs and re-establish cytoplasmic/protoplasmic flow in injured hyphae. When the HHM is completed, we suggest that other GHTs and their ramifications become obsolete and cytoplasmic/protoplasmic material is saved. According to Kokkoris et al. (2020), it is presumed that AMF retract cytoplasm and nuclei back into the spore, by the presence of "retention septa" in the hyphae, to preserve them possibly as a resource. In several AMF species (*Funneliformis mosseae*, *Gi. margarita*, and *Claroideoglomus etunicatum*) arrest of hyphal growth was observed in the absence of roots followed by the formation of septa with cytoplasmic/protoplasmic retraction (Logi et al. 1998). This mechanism is thus not only associated with mycelial senescence and aging but possibly also with control of energy resources in the fungus (i.e., spores) (Logi et al. 1998). This mechanism also was noticed in *R. irregularis*, presenting retraction in all treatments.

This study showed that the four events leading to complete hyphae healing, described by de la Providencia et al. (2005) in the absence of pollutant, were observed in its presence. For both fungi, these steps were delayed in the presence of diesel, but appeared less affected in *Gigaspora* sp. than in *R. irregularis*, supporting our initial hypothesis. In *Gigaspora* sp., the hyphae in most cases were able to heal following injury at a similar frequency as observed in absence of diesel. This was the most likely way to maintain the viability of the hyphae in adverse conditions, and represents a mechanism for mycelia to survive. Conversely, in *R. irregularis*, the limited healing of severed hyphae, but the capacity of the GHTs developed to anastomose with neighboring hyphae or even contact roots (de la Providencia et al. 2005, 2007) could increase the chances of injured hyphae to maintain a connected network. These modes of HHM are key for AMF ecology and for AMF application in phytoremediation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00572-021-01026-5.

Funding This research was sponsored by the CIUF–CUD (currently ARES, Académie de Recherche et d'enseignement Supérieur Wallonie-Bruxelles, Commission de la Coopération pour le Dévelopment CIUF) through the PIC Project entitled "Reinforcement of the fungal expertise in Ecuador via case studies of fungal plants interactions in selected ecosystems and the development of biotechnology-oriented fungal resource." Additional support was given to Mónica Garcés Ruiz by the Pontificia Universidad Católica del Ecuador (PUCE) projects.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Abha S, Singh CS (2012) Hydrocarbon pollution: effects on living organisms, remediation of contaminated environments, and effects of heavy metals co-contamination on bioremediation. Introd to Enhanc Oil Recover Process Bioremediat Oil-Contaminated Sites 21:185–206. https://doi.org/10.5772/48014

Adam G, Duncan H (1999) Effect of diesel fuel on growth of selected plant species. Environ Geochem Health 21:353–357

Amadi A, Abbey SD, Nma A (1996) Chronic effects of oil spill on soil properties and microflora of a rainforest ecosystem in Nigeria. Water Air Soil Pollut 86:1–11. https://doi.org/10.1007/BF00279142

Cabello MN (1999) Effectiveness of indigeneous arbuscular mycorrhizal fungi (AMF) isolated from hydrocarbon polluted soils. Basic Microbiol 39:89–95

Calonne-Salmon M, Plouznikoff K, Declerck S (2018) The arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 increases the phosphorus uptake and biomass of *Medicago truncatula*, a benzo[a]pyrene-tolerant plant species. Mycorrhiza 28:761–771. https://doi.org/10.1007/s00572-018-0861-9

Calonne M, Fontaine J, Debiane D et al (2014a) Impact of anthracene on the arbuscular mycorrhizal fungus lipid metabolism1. Botany 92:173–178. https://doi.org/10.1139/cjb-2013-0094

Calonne M, Fontaine J, Debiane D et al (2014b) The arbuscular mycorrhizal *Rhizophagus irregularis* activates storage lipid biosynthesis to cope with the benzo[a]pyrene oxidative stress. Phytochemistry 97:30–37. https://doi.org/10.1016/j.phytochem.2013.10.014

Cox DR (1972) Regression Models and Life-Tables. J R Stat Soc Ser B 34:187–202. https://doi.org/10.1111/j.2517-6161.1972.tb00899.x

Cranenbrouck S, Voets L, Bivort C et al (2005) Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck S, Strullu D, Fortin J (eds) In vitro culture of mycorrhizas. Springer, Berlin Heidelberg, Berlin, pp 341–348

Dalpé Y, Trépanier M, Lounès-Hadj Sahraoui A et al (2012) Lipids of Mycorrhizas. In: Hock B (ed) Fungal Associations, The Mycota IX, 2nd edn. Springer-Verlag, Berlin Heidelberg, pp 137–169

de la Providencia IE, de Souza FA, Fernández F et al (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271. https://doi.org/10.1128/AEM.69.7.4308-4311.2003

de la Providencia IE, Fernández F, Declerck S (2007) Hyphal healing mechanism in the arbuscular mycorrhizal fungi *Scutellospora reticulata* and *Glomus clarum* differs in response to severe physical stress. FEMS Microbiol Lett 268:120–125. https://doi.org/10.1111/j.1574-6968.2006.00572.x

de Novais CB, Avio L, Giovannetti M et al (2019) Interconnectedness, length and viability of arbuscular mycorrhizal mycelium as affected by selected herbicides and fungicides. Appl Soil Ecol 143:144–152. https://doi.org/10.1016/j.apsoil.2019.06.013

de Novais CB, Pepe A, Siqueira JO et al (2017) Compatibility and incompatibility in hyphal anastomosis of arbuscular mycorrhizal fungi. Sci Agric 74:411–416. https://doi.org/10.1590/1678-992x-2016-0243

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012. https://doi.org/10.1080/15572536.2004.11833016

Debiane D, Calonne M, Fontaine J et al (2011) Lipid content disturbance in the arbuscular mycorrhizal, *Glomus irregulare* grown in monoxenic conditions under PAHs pollution. Fungal Biol 115:782–792. https://doi.org/10.1016/j.funbio.2011.06.003

Debiane D, Garçon G, Verdin A et al (2008) In vitro evaluation of the oxidative stress and genotoxic potentials of anthracene on mycorrhizal chicory roots. Environ Exp Bot 64:120–127. https://doi.org/10.1016/j.envexpbot.2008.04.003

Debiane D, Garçon G, Verdin A et al (2009) Mycorrhization alleviates benzo[a]pyrene-induced oxidative stress in an in vitro chicory root model. Phytochemistry 70:1421–1427. https://doi.org/10.1016/j.phytochem.2009.07.002

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585. https://doi.org/10.2307/3761216

Delord M (2015) MIICD: data augmentation and multiple imputation for interval censored data. R Libr. https://doi.org/10.13140/RG.2.1.2084.8168

Dorn PB, Salanitro JP (2000) Temporal ecological assessment of oil contaminated soils before and after bioremediation. Chemosphere 40:419–426. https://doi.org/10.1016/S0045-6535(99)00304-5

Driai S, Verdin A, Laruelle F et al (2015) Is the arbuscular mycorrhizal fungus *Rhizophagus irregularis* able to fulfil its life cycle in the presence of diesel pollution? Int Biodeterior Biodegrad 105:58–65. https://doi.org/10.1016/j.ibiod.2015.08.012

Faure D, Bonin P, Duran R (2015) Environmental microbiology as a mosaic of explored ecosystems and issues. Environ Sci Pollut Res 22:13577–13598. https://doi.org/10.1007/s11356-015-5164-5

Fontaine J, Grandmougin-Ferjani A, Glorian V, Durand R (2004) 24-Methyl/methylene sterols increase in monoxenic roots after colonization by arbuscular mycorrhizal fungi. New Phytol 163:159–167. https://doi.org/10.1111/j.1469-8137.2004.01075.x

Franco-Ramírez A, Ferrera-Cerrato R, Varela-Fregoso L et al (2007) Arbuscular mycorrhizal fungi in chronically petroleum-contaminated soils in Mexico and the effects of petroleum hydrocarbons on spore germination. J Basic Microbiol 47:378–383

Gan S, Lau EV, Ng HK (2009) Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). J Hazard Mater 172:532–549. https://doi.org/10.1016/j.jhazmat.2009.07.118

George B, Seals S, Aban I (2014) Survival analysis and regression models. J Nucl Cardiol 21:686–694. https://doi.org/10.1007/s12350-014-9908-2

Giovannetti M, Avio L, Sbrana C (2015) Functional significance of anastomosis in arbuscular mycorrhizal networks. In: Horton TR (ed) Mycorrhizal Networks. Ecological Studies, Springer Science+Business, pp 41–68

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Fortuna P, Citernesi AS et al (2001) The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol 151:717–724. https://doi.org/10.1046/j.0028-646x.2001.00216.x

Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. New Phytol 164:175–181. https://doi.org/10.1111/j.1469-8137.2004.01145.x

IJdo M, Cranenbrouck S, Declerck S (2011) Methods for large-scale production of AM fungi: past, present, and future. Mycorrhiza 21:1–16

ISO (International-Organization-for-Standardization) (2009) ISO/TS 10832: Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test. In: ISO/TS 10832. https://www.iso.org/obp/ui/#iso:std:iso:ts:10832:ed-1:v2:en. Accessed 27 Jul 2020

Joner EJ, Leyval C (2001) Influence of arbuscular mycorrhiza on clover and ryegrass grown together in a soil spiked with polycyclic aromatic hydrocarbons. Mycorrhiza 10:155–159. https://doi.org/10.1007/s005720000071

Joye SB, Teske AP, Kostka JE (2014) Microbial dynamics following the macondo oil well blowout across gulf of Mexico environments. Bioscience 64:766–777. https://doi.org/10.1093/biosci/biu121

Kauppi S, Sinkkonen A, Romantschuk M (2011) Enhancing bioremediation of diesel-fuel-contaminated soil in a boreal climate: Comparison of biostimulation and bioaugmentation. Int Biodeterior Biodegrad 65:359–368. https://doi.org/10.1016/j.ibiod.2010.10.011

Kirk JL, Moutoglis P, Klironomos J et al (2005) Toxicity of diesel fuel to germination, growth and colonization of *Glomus intraradices* in soil and in vitro transformed carrot root cultures. Plant Soil 270:23–30. https://doi.org/10.1007/s11104-004-1013-x

Kirk P, Cannon PF, David J, Stalpers J (2001) Ainsworth and Bisby's Dictionary of the Fungi, 9th edn. CABI, Wallingford, UK

Kokkoris V, Stefani F, Dalpé Y et al (2020) Nuclear dynamics in the arbuscular mycorrhizal fungi. Trends Plant Sci 25:765–778. https://doi.org/10.1016/j.tplants.2020.05.002

Lenoir I, Fontaine J, Tisserant B et al (2017) Beneficial contribution of the arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, in the protection of *Medicago truncatula* roots against benzo[a] pyrene toxicity. Mycorrhiza 27:465–476. https://doi.org/10.1007/s00572-017-0764-1

Lenoir I, Lounes-Hadj Sahraoui A, Fontaine J (2016) Arbuscular mycorrhizal fungal-assisted phytoremediation of soil contaminated with persistent organic pollutants: a review. Eur J Soil Sci 67:624–640. https://doi.org/10.1111/ejss.12375

Leyval C, Binet P (1998) Effect of polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants. J Environ Qual 27:402–407. https://doi.org/10.2134/jeq1998.00472425002700020022x

Liang F, Lu M, Keener TC et al (2005) The organic composition of diesel particulate matter, diesel fuel and engine oil of a non-road diesel generator. J Environ Monit 7:983–988. https://doi.org/10.1039/b504728e

Logi C, Sbrana C, Giovannetti M (1998) Cellular events involved in survival of individual arbuscular mycorrhizal symbionts growing in the absence of the host. Appl Environ Microbiol 64:3473–3479

Meglouli H, Fontaine J, Verdin A et al (2019) Aided phytoremediation to clean up dioxins/furans-aged contaminated soil: Correlation between microbial communities and pollutant dissipation. Microorganisms 7:1–18. https://doi.org/10.3390/microorganisms7110523

Merkl N, Schultze-Kraft R, Infante C (2005) Phytoremediation in the tropics—influence of heavy crude oil on root morphological characteristics of graminoids. Environ Pollut 138:86–91. https://doi.org/10.1016/j.envpol.2005.02.023

Nicolotti G, Egli S (1998) Soil contamination by crude oil: Impact on the mycorrhizosphere and on the revegetation potential of forest trees. Environ Pollut 99:37–43. https://doi.org/10.1016/S0269-7491(97)00179-6

Nwoko CO (2014) Effect of arbuscular mycorrhizal (AM) fungi on the physiological performance of *phaseolus vulgaris* grown under crude oil contaminated soil. J Geosci Environ Prot 2:9–14. https://doi.org/10.4236/gep.2014.24002

Peng M, Zi X, Wang Q (2015) Bacterial community diversity of oil-contaminated soils assessed by high throughput sequencing of 16s rRNA genes. Int J Environ Res Public Health 12:12002–12015. https://doi.org/10.3390/ijerph121012002

Plouznikoff K, Declerck S, Calonne-Salmon M (2016) Mitigating abiotic stresses in crop plants by arbuscular mycorrhizal fungi. In: Vos CMF, Kazan K (eds) Belowground Defence Strategies in Plants. Springer International Publishing Switzerland, Signaling and Communication in Plants, pp 341–400

Purin S, Morton JB (2011) In situ analysis of anastomosis in representative genera of arbuscular mycorrhizal fungi. Mycorrhiza 21:505–514. https://doi.org/10.1007/s00572-010-0356-9

Rajtor M, Piotrowska-Seget Z (2016) Prospects for arbuscular mycorrhizal fungi (AMF) to assist in phytoremediation of soil hydrocarbon contaminants. Chemosphere 162:105–116. https://doi.org/10.1016/j.chemosphere.2016.07.071

Salami AO, Elum EA (2010) Bioremediation of a crude oil polluted soil with *Pleurotus pulmonarius* and *Glomus mosseae* using *Amaranthus hybridus* as a test plant. J Bioremediation Biodegrad 01. https://doi.org/10.4172/2155-6199.1000113

Sbrana C, Fortuna P, Giovannetti M (2011) Plugging into the network: belowground connections between germlings and extraradical mycelium of arbuscular mycorrhizal fungi. Mycologia 103:307–316. https://doi.org/10.3852/10-125

Simard SW, Beiler KJ, Bingham MA et al (2012) Mycorrhizal networks: mechanisms, ecology and modelling. Fungal Biol Rev 26:39–60. https://doi.org/10.1016/j.fbr.2012.01.001

Stefani FOP, Bell TH, Marchand C et al (2015) Culture-dependent and -independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. PLoS One 10:e0128272. https://doi.org/10.1371/journal.pone.0128272

Tang M, Chen H, Huang JC, Tian ZQ (2009) AM fungi effects on the growth and physiology of *Zea mays* seedlings under diesel stress. Soil Biol Biochem 41:936–940. https://doi.org/10.1016/j.soilbio.2008.11.007

Vanden Bossche H (1990) Importance and role of sterols in fungal membranes. In: Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW, Copping LG (ed) Biochemistry of Cell Walls and Membranes in Fungi. Springer-Verlag, Berlin, pp 135–157

Verdin A, Lounès-Hadj Sahraoui A, Fontaine J et al (2006) Effects of anthracene on development of an arbuscular mycorrhizal fungus and contribution of the symbiotic association to pollutant dissipation. Mycorrhiza 16:397–405. https://doi.org/10.1007/s00572-006-0055-8

Voets L, de la Providencia IE, Declerck S (2006) Glomeraceae and Gigasporaceae differ in their ability to form hyphal networks. New Phytol 172:185–188. https://doi.org/10.1111/j.1469-8137.2006.01873.x

Voets L, de la Providencia IE, Fernandez K et al (2009) Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under in vitro conditions. Mycorrhiza 19:347–356. https://doi.org/10.1007/s00572-009-0233-6

Vwioko DE, Omamogho CE (2012) Assessing successive plant growth on petroleum hydrocarbon degradation in highly polluted soil augmented with wood ash. Int J Appl Sci Technol 2:247–267

Weete JD (1989) Structure and function of sterols in fungi. Adv Lipid Res 23:115–167