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RESEARCH

The systemic herbicide glyphosate affects the sporulation dynamics of *Rhizophagus* species more severely than mechanical defoliation or the contact herbicide diquat

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Abstract
Arbuscular mycorrhizal fungi (AMF) are totally dependent on a suitable host plant for their carbon resources. Here, we investigated under in vitro conditions, the impact of defoliation practices, i.e., mechanical defoliation or chemical defoliation with a contact herbicide (Reglone®, containing the active ingredient diquat) or systemic herbicide (RoundUp®, containing the active ingredient glyphosate), on the dynamics of spore production of *Rhizophagus irregularis* and *Rhizophagus intraradices* associated with *Solanum tuberosum* and/or *Medicago truncatula*. Glyphosate affected the spore production rate more rapidly and severely than diquat or mechanical defoliation. We hypothesize that this effect was related to disruption of the C metabolism in the whole plant combined with a possible direct effect of glyphosate on the fungus within the roots and/or perhaps in soil via the release of this active ingredient from decaying roots. No glyphosate could be detected in the roots due to technical constraints, while its release from the roots in the medium corresponded to 0.11% of the active ingredient applied to the leaves. The three defoliation practices strongly affected root colonization, compared to the non-defoliated plants. However, the amount of glyphosate released into the medium did not affect spore germination and germ tube growth. These results suggest that the effects of defoliation on the dynamics of spore production are mainly indirect via an impact on the plant, and that the effect is faster and more marked with the glyphosate-formulation, possibly via a direct effect on the fungus in the roots and more unlikely on spore germination.

Keywords Arbuscular mycorrhizal fungi · *Solanum tuberosum* · *Medicago truncatula* · Defoliant · Germ tube length · Root colonization

Introduction

Arbuscular mycorrhizal fungi (AMF) colonize the roots of most crops and weeds without distinction (Yamato 2004; Wipf et al. 2019; El Omari and El Ghachtouli 2021). They supply plants with nutrients (e.g., N and P) in exchange for photosynthetic products (between 0.10% and 11.40% of net primary productivity) in the form of sugars and fatty acids (Hawkins et al. 2023), which represent a significant cost for the host plant (Kiers and van der Heijden 2006; Douglas 2008; Giovannini et al. 2020; El Omari and El Ghachtouli 2021). As these fungi are entirely dependent on C sources derived from photosynthesis, they are unable to complete their life cycle without a living host (Bécard et al. 2004; Wipf et al. 2019). Mechanical defoliation or defoliation using either a contact or systemic chemical herbicide, three common agricultural practices used to facilitate crop harvesting, suppress cover crops or control weeds, are therefore likely to have an impact on AMF.

Mechanical defoliation (Saravesi et al. 2014; Ambrosino et al. 2020) or severe grazing by herbivores (Gehring and Whitham 1994; Yang et al. 2020) have often been reported to affect root colonization by AMF, suggesting that it is limited by host C availability. However, in contrast a number of studies have reported increased root colonization following grazing (Barto and Rillig 2010; van der Heyde et al. 2017). Similarly, a decrease in spore production has

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been observed immediately after mechanical defoliation (IJdo et al. 2010; Saravesi et al. 2014; Kusakabe et al. 2018), although an increase also has been mentioned (Klironomos et al. 2004; Tawaraya et al. 2012). The differences observed in root colonization and sporulation after defoliation among studies may be related to various factors (as reviewed by Barto and Rillig (2010)) including fungal species and life history strategy (LHS). For example, IJdo et al. (2010) demonstrated in vitro, that repeated defoliation of *Medicago truncatula* affected *Rhizophagus intraradices* (a r-strategist) and *Scutellospora reticulata* (a K-strategist) differently. The spore production rate of *R. intraradices* decreased immediately after defoliation and recovered shortly afterwards when the leaves regrew, while *S. reticulata* showed greater resistance to disturbance over time (IJdo et al. 2010). Klironomos et al. (2004) also found that the effects of defoliation differed between AMF species and depended on the frequency of defoliation.

A direct impact of chemical herbicides on AMF (e.g., spore viability and germination, plant colonization) has been reported in several studies (Pasaribu et al. 2011; Druille et al. 2013a; Li et al. 2013; Karpouzas et al. 2014; Helander et al. 2018), but none, to our knowledge, have reported their effects on spore production dynamics and only few have mentioned an indirect impact, via the plant, on root colonization (Zaller et al. 2014; Hage-Ahmed et al. 2019).

Glyphosate (Monsanto, Belgium) is the world's most widely used non-selective – toxic for all plants – systemic herbicide used to control weeds or suppress cover crops (Woodburn 2000; Duke 2021). It kills plants by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), a key enzyme in the shikimate biosynthetic pathway (Tzin et al. 2012; Helander et al. 2012; Tall and Puigbò 2020). Over 30% of the C fixed by plants passes through this pathway (Shaner 2006; Tzin et al. 2012). As a systemic herbicide, glyphosate is translocated from the treated leaves to shoots and roots within 48 to 72 h (Wakelin et al. 2004; Eker et al. 2006). Neumann et al. (2006) showed that glyphosate was then released into the soil through root decomposition, possibly affecting non-target microorganisms (e.g., fungi or bacteria) in the roots or rhizosphere, modifying microbial communities, and thus potentially affecting ecosystem functions (Araújo et al. 2003; Kanissery et al. 2019). The persistence and transport of glyphosate in soil depend on soil composition, climatic conditions and microbial activity (Carlisle and Trevors 1988; Bai and Ogbourne 2016; Bento et al. 2016), as well as agricultural practices (Helander et al. 2012). Studies conducted in vitro (Wan et al. 1998) and in vivo (Druille et al. 2013b, 2016) have shown that glyphosate and aminomethylphosphonic acid (AMPA), a secondary metabolite of glyphosate, are relatively toxic to AMF (IC_{50} of 0.5 ± 0.3 ppm and 3.8 ± 2.8 ppm, respectively). For example, Druille et al. (2013b) showed a marked reduction in arbuscules with glyphosate applied to plant leaves.

Diquat (Syngenta Crop Protection, Belgium) is a fast-acting, non-selective contact herbicide inhibiting photosynthesis by diverting the flow of electrons from photosystem I with subsequent inhibition of $NADP^+$ reduction and production of peroxide radicals leading to destruction of cell membranes (Zweig et al. 1965; Dodge and Harris 1970). It is used to dry many crops to facilitate seed harvesting. As a contact herbicide, spraying it on plant leaves will inhibit photosynthesis and thus cause plant death. To our knowledge, no study on the effects of diquat on AMF has been conducted so far. It is likely that under natural conditions diquat has no direct impact on AMF because it is not translocated to roots (Smith et al. 1981) and is inactivated and immobilized in the soil (Tucker et al. 1967). Conversely, it can reduce AMF spore production by affecting plant photosynthesis and hence fixed carbon.

In this study, the objective was to evaluate, under in vitro culture conditions, the impact of different defoliation practices on the spore production dynamics of *Rhizophagus irregularis* MUCL 41833 and *Rhizophagus intraradices* MUCL 49410. In Experiment 1, we assessed and compared the effects of mechanical defoliation with defoliation by a contact chemical herbicide (Reglone® from Syngenta Crop Protection N.V., Belgium, containing the active ingredient diquat) and a systemic chemical herbicide (RoundUp® Max from Monsanto, Belgium, containing the active ingredient glyphosate), on the spore production dynamics of the two AMF. Spores were considered because their production is strongly linked to mycelium production (Declerck et al. 2001) and they are considered to be C storage structures in the mycelium as shown by radioactive tracers (Bago et al. 2003). Spores also are the easiest fungal structures to observe and quantify dynamically and non-destructively in vitro (Declerck et al. 2001; IJdo et al. 2010). Finally, spore production by *R. intraradices* is influenced instantaneously by mechanical defoliation (IJdo et al. 2010). This treatment, therefore, represents a suitable control for evaluating and comparing the short-term effects of mechanical defoliation with those of a contact and a systemic herbicide on AMF. The study was carried out using *Solanum tuberosum* and *Medicago truncatula* as model plants. In Experiment 2, we quantified the release of the active ingredient glyphosate into the medium, after application of RoundUp® to the leaves and shoots of *Solanum tuberosum*. In Experiment 3, the impact of different glyphosate concentrations was assessed on spore germination and germ tube length of *R. intraradices*.

Materials and methods

Biological material

Potato (*Solanum tuberosum* L., cv. Bintje was provided by the Station de Haute Belgique (Libramont, Belgium)). The

plants were genetically identical and micro-propagated every 5 weeks. Briefly, 10 nodal cuttings were placed in sterile microbox containers (90 × 120 mm, size: 870 mL, Sac O_2, Belgium) containing 100 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 20 g L^{-1} sucrose (Duchefa, The Netherlands), 3 g L^{-1} Phytagel™ (Sigma-Aldrich, Merck KGaA, Germany) and adjusted to pH 5.9 before sterilization (121 °C for 15 min). The plants were maintained in a growth chamber under controlled conditions (i.e., 22 °C/18 °C (day/night), 70% relative humidity (RH), a photoperiod of 16 h day $^{-1}$ and a photosynthetic photon flux density (PPFD) of 225 µmol m^{-2} s^{-1}), until use.

Seeds of barrel medic (*Medicago truncatula* Gaertn., cv. Jemalong A 17 (SARDI, Australia)) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed in sterilized (121 °C for 15 min) deionized water. Seeds were germinated in Petri plates (90 mm diameter) containing 35 mL of Modified Strullu-Romand (MSR) medium (Declerck et al. 1998) without sucrose and vitamins, solidified with 3 g L^{-1} Phytagel™ and adjusted to pH 5.5 before sterilization (121˚C for 15 min). The Petri plates were incubated at 27˚C in the dark. Seedlings were used 4 days after germination.

Rhizophagus irregularis (Błaszk., Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] MUCL 41833 and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler (2010) MUCL 49410 were supplied by the Glomeromycota in vitro collection (GINCO, Belgium) and maintained in vitro in bi-compartmented Petri plates (St-Arnaud et al. 1996) until thousands of spores were produced. *R. irregularis* and *R. intraradices* were selected because of their ubiquitous distribution and occurrence in agricultural soils. Moreover, these strains are extensively studied under in vitro conditions (Declerck et al. 2001; Gil-Cardeza et al. 2017; Le Pioufle and Declerck 2018). Both strains (formerly classified as *Glomus* species) have similar spore production dynamics with lag, log and plateau phases (Declerck et al. 1996, 2001).

Chemicals

Herbicides

Two herbicides were used: RoundUp® Max (Monsanto, Belgium), a systemic herbicide containing 450 g L^{-1} glyphosate, diluted in 250 mL of water (8 mL L^{-1}) which corresponds to the field dose (0.72 kg ha^{-1}) (Authority (EFSA) 2015) and Reglone® (Syngenta Crop Protection N.V., Belgium), a contact herbicide containing 200 g L^{-1} diquat, diluted in 250 mL of water (1.5 mL L^{-1}) which is lower than the recommended field dose (5 L ha^{-1}) (Reglone—SYNGENTA Herbicides, n.d.), but was determined in a preliminary experiment to be the minimum dose necessary to completely dry plant leaves after 96 h (data not shown). They are referred to as glyphosate-formulation (Glyphosate-F) and diquat-formulation (Diquat-F), respectively, in the rest of this report.

Chemicals for glyphosate quantification

The following chemicals were used for Experiment 2. Organic solvents (methanol (MeOH) and acetonitrile (ACN) (VWR International, Belgium)) of HPLC grade were used for the quantification of glyphosate. Purified and demineralized water was obtained with a Milli-Q system (Avidity ALTOi). Glyphosate and aminomethylphosphonic acid (AMPA) were purchased from Sigma-Aldrich (Sigma-Aldrich, Merck KGaA, Germany) and used to prepare standard solutions for glyphosate quantification. Glyphosate powder also was used to obtain solutions to study the effects of glyphosate on AMF spore germination. Boric acid (H_3BO_3) (Merck, Germany), potassium chloride (KCl) (Merck, Germany) and sodium hydroxide 1 M (NaOH) (VWR Chemicals, Avntor) were employed to prepare borate buffer pH 9. Borate buffer and 9-fluorenylmethylchloroformate (FMOC-Cl) (Sigma-Aldrich, Merck KGaA, Germany) were used for the derivatization. Phosphoric acid (H_3PO_4, 85%) (AROS Organics), NaOH (50%) (VWR Chemicals, Avantor) and sodium nitrate (NaN_3) (Merck, Germany) were used to prepare phosphate buffer 10 mM pH 7 used as the mobile phase.

Experimental procedures

Exp. 1: Impact of mechanical or chemical (with contact or systemic herbicides) defoliation on the spore production dynamics of *R. irregularis* and *R. intraradices* associated with *S. tuberosum* or *M. truncatula*

Design of the in vitro *culture system*

Ten-day-old rooted nodal cuttings of potato or 4-day-old seedlings of barrel medic were transferred to single-compartmented Petri plates (90 mm diam.) containing 40 mL of MSR medium (Declerck et al. 1998) free from sucrose and vitamins, solidified with 3 g L^{-1} Phytagel™ (Sigma-Aldrich, Merck KGaA, Germany) and adjusted to pH 5.5 before being sterilized (121˚C for 15 min). Roots were placed on the MSR medium and the shoot protruded in the open air through an opening (± 2 mm diam.) made in the base and lid of the Petri plates (Online Resource 1). Approximately 100 spores of *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410 were inoculated in the vicinity of the roots. Each Petri plate was then closed and sealed with Parafilm M® (Bemis Company, Inc., USA) and the opening was carefully plastered with sterilized (121 °C for 15 min) silicon grease (VWR

International, Belgium) to avoid contamination. The Petri plates were covered with opaque plastic bags to keep the AMF and plant roots in the dark. They were further incubated in a growth chamber under controlled conditions (i.e., 22/18 °C (day/night), 70% RH, a photoperiod of 16 h day^{-1} and an average PPFD of 225 µmol m^{-2} s^{-1}). MSR medium was added every two weeks to the potato and once a week to the barrel medic plant systems. After defoliation, MSR medium was added only to the "Control" treatments (no defoliation) because defoliated plants did not need extra medium.

Experimental setup

A profuse mycelium bearing numerous spores was obtained after a few weeks (five for potato, eight or nine for barrel medic) of association with the AMF. For potato plants associated with *R. irregularis* MUCL 41833 and barrel medic plants associated with *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410, the systems were divided into four groups (Table 1) in such a way that the average number of spores at the time of treatment was similar for all groups (Table 2). The first group of plants was not defoliated ("Control"). The three other groups were defoliated either mechanically with scissors ("Mechanical") or chemically ("Diquat-F" and "Glyphosate-F").

For potato, the four groups (each with 11 systems) were used in a single experiment with *R. irregularis* MUCL 41833. For barrel medic, a first trial was performed with the Control and Glyphosate-F groups (with 5 systems for each group), with *R. irregularis* MUCL 41833 strain, and a second trial (Experiment 1 comprised both trials) was performed with the Control (6 systems), Diquat-F (7 systems), and Mechanical (8 systems) groups with *R. intraradices* strain MUCL 49410 (Table 1). The dynamics of spore production over the first 8 weeks of association, i.e., before defoliation treatment, were similar for plants

Table 1 Number of replicated autotrophic in vitro culture systems considered within each treatment (Control, Mechanical, Glyphosate-F or Diquat-F) and for each plant-AMF association (Potato- *R. irregularis*, Barrel medic – *R. irregularis*, Barrel medic – *R. intraradices*) for Experiment 1 and within the Control or Glyphosate-F treatments for each plant (Potato – Barrel medic) for Experiment 2. Contaminations and dead plant material reduced the number of replicates among treatments

AMF strain Plant		**Experiment 1**			**Experiment 2**
		R. irregularis		*R. intraradices*	*No AMF*
		Potato	Barrel medic	Barrel medic	Potato
Treatments	Control	11	5	6	4
	Mechanical	11	NA	8	
	Glyphosate-F	11	5	NA	18
	Diquat-F	11	NA	7	

Table 2 AMF growth descriptors measured, in Exp. 1, in the autotrophic in vitro culture systems according to three treatment groups (Mechanical, Diquat-F and Glyphosate-F) and a non-defoliated control with potato and barrel medic plants: number of replicate systems followed through (n), mean number of spores at defoliation (time 0), root dry weight (DW) and intra-radical colonization levels (presented as Total%, Arbuscules% and Spores/Vesicles%) at harvest (8 weeks and 6 weeks after defoliation for potato and barrel medic, respectively)

Experimental design				At defoliation (time 0)	Root colonization			
Plant	**Treatment**	**N**	**AMF strain**	**Number of spores**	**Root DW (g)**	**Total (%)**	**Arbuscules (%)**	**Spores/Vesicles (%)**
					At harvest (8 weeks after defoliation)			
Potato	Control	11	*R. irregularis*	311 ± 68^{a}	0.079 ± 0.007^{a}	41.2 ± 2.0^{a}	9.4 ± 1.1^{a}	3.8 ± 0.5^{a}
	Mechanical	11	*R. irregularis*	300 ± 81^{a}	0.054 ± 0.003^{b}	16.5 ± 2.3^{b}	2.6 ± 0.7^{b}	1.1 ± 0.4^{b}
	Diquat-F	11	*R. irregularis*	292 ± 69^{a}	0.060 ± 0.003^{b}	19.8 ± 2.0^{b}	3.1 ± 0.6^{b}	1.0 ± 0.3^{b}
	Glyphosate-F	11	*R. irregularis*	304 ± 78^{a}	0.055 ± 0.003^{b}	19.0 ± 2.2^{b}	2.1 ± 0.3^{b}	0.8 ± 0.2^{b}
					At harvest (6 weeks after defoliation)			
Barrel medic	Control	11	*R. irregularis & R. intraradices*	929 ± 218^{a}	0.132 ± 0.002^{a}	64.8 ± 1.6^{a}	12.4 ± 0.3^{a}	6.7 ± 0.3^{a}
	Mechanical	8	*R. intraradices*	1157 ± 305^{a}	0.086 ± 0.002^{b}	35.4 ± 2.3^{b}	5.8 ± 0.2^{c}	3.0 ± 0.2^{b}
	Diquat-F	7	*R. intraradices*	1226 ± 355^{a}	0.088 ± 0.002^{b}	35.7 ± 1.8^{b}	7.2 ± 0.2^{b}	3.4 ± 0.2^{b}
	Glyphosate-F	5	*R. irregularis*	840 ± 284^{a}	0.074 ± 0.004^{c}	35.1 ± 0.8^{b}	5.4 ± 0.3^{c}	1.5 ± 0.3^{c}

The parameters measured are expressed as mean ± standard error (SE). Different letters indicate significant differences (Tukey's HSD, $\alpha = 0.05$)

inoculated in the two experiments, i.e., with *R. irregularis* MUCL 41833 and with *R. intraradices* MUCL 49410 (Fig. 1). No significant difference in spore abundance eight weeks after association was observed between *R. irregularis* and *R. intraradices* (one-way ANOVA, $F_{1,29}=3.00$, $p=0.094$), allowing the results of the two experiments to be pooled for the statistical analysis of spore production dynamics. Mechanical defoliation was performed with scissors at the crown of the plant. Diquat-F and Glyphosate-F were applied homogeneously on the shoots and leaves of each plant with a manual sprayer. Defoliation was performed once and no regrowth was observed.

Spore production dynamics

The spore production dynamics were followed weekly under a stereo-microscope (Olympus SZ40, Olympus Optical GmbH, Germany) at 10 to 40 × magnification. A grid of lines was marked on the bottom of each autotrophic culture system to facilitate counting (Declerck et al. 1996). Quantification was regularly performed before and after the application of the defoliation treatment (Fig. 1).

Spore production dynamics were evaluated by quantifying the average weekly rate of spore production from defoliation (R_t) according to the following formula:

$$R_t = \sqrt[t]{\frac{N_t}{N_0}}$$

with N_t being the abundance of spores at week t, and N_0 the abundance of spores when the defoliation treatment was applied (week 0). Density dependence is known to affect spore production rate (Declerck et al. 2001), but the autotrophic culture systems were distributed among the four treatment groups in such a way that they had a similar average abundance of spores at the week of defoliation (Table 2). We decided therefore not to implement any correction for density dependence, our analysis being conservative because the effect of density dependence, if any, would be randomized among the four treatment groups and increase the residual variation, i.e., making the effects less significant statistically.

The spore production rate was ln-transformed and analyzed, for each plant species separately, using a three-way ANOVA model (time and defoliation as fixed crossed factors, replicate as random factor crossed with time but nested within defoliation). Differences between defoliation types were subsequently tested week by week using contrasts to establish the rapidity of the impact of each defoliation treatment. The number of replicates per treatment being different in the experiment with barrel medic (Table 2), we checked that the results of the ANOVA analysis were robust to this imbalance. To do so, we randomly down-sampled the data to produce a series of 1000 perfectly balanced datasets (with 5 replicate systems per defoliation) and recomputed the ANOVA tests.

Root weight and colonization by AMF

At the end of the experiment, roots were harvested and AMF colonization was estimated. Roots were first cleaned of MSR medium and then dried at 60 °C for 24 h to estimate dry weight. The roots subsequently were cleared with 10% KOH at 50 °C for 90 min and stained with 5% blue ink (Parker®) diluted in vinegar (7° acidity) at 50 °C. The roots were rinsed with deionized water and observed under a compound microscope (Olympus BH2, Olympus Optical GmbH, Germany) at × 200 magnification. Root colonization was assessed by evaluating the percentages of total root colonization, arbuscules and vesicles/spores in 200 microscopic intersects, following the method of McGonigle et al. (1990). These variables were normalized by arcsine transformation and the effect of defoliation was tested using a one-way ANOVA, followed by Tukey's honest significant difference tests to compare individual defoliations.

Exp. 2: Analysis of glyphosate and AMPA residues in culture medium and roots of *Solanum* tuberosum after foliar application of glyphosate

Design of the in vitro *culture system*

Ten-day-old rooted nodal cuttings of potato were transferred to sterile pots (60 × 45 × 75 mm, size: 125 mL, MLS nv, Belgium) filled with 50 mL of MSR medium without sucrose and vitamins, solidified with 3 g L^{-1} Phytagel™ (Sigma-Aldrich, Merck KGaA, Germany) and adjusted to pH 5.5 before sterilization (121 °C for 15 min). A sterile 41 µm nylon mesh (PROSEP BVBA, Belgium) was deposited on the MSR medium to avoid roots of the plants penetrating the medium. The pot lid was pierced in the middle by a hole (± 2 mm diam.) through which the shoot was allowed to grow, while the roots developed in the open space inside the pots and in contact with the mesh, accessing nutrients by capillarity. To avoid contamination, the holes were carefully plastered with sterilized (121 °C for 15 min) silicon grease (VWR International, Belgium) and each system was sealed with Parafilm M® (Bemis Company, Inc., USA) (Online Resource 2). The systems were further incubated in a growth chamber under controlled conditions (i.e., 22/18 °C (day/night), 70% RH, a photoperiod of 16 h day^{-1} and an average PPFD of 225 µmol m^{-2} s^{-1}).

Before Glyphosate-F application (i.e., after five weeks of growth of potato), plantlets were transferred to new systems without nylon mesh, filled with 50 mL of sterilized water (121 °C for 15 min) (Online Resource 2). It enabled the development of the roots inside the sterile water and the exudation of glyphosate into the water following its application

on the leaves and diffusion to the shoots and roots. This system allowed for the detection of glyphosate residues released into the water.

Experimental setup

The plant systems filled with water were divided into two groups (Table 1). Plants in the first group received no glyphosate ("Control"). Plants in the second group received an application of 2 mL of the herbicide ("Glyphosate-F") by spraying the shoots and leaves of each plant over a container (to collect the excess). The plants were left under a chemical hood until they died.

Quantitative analysis of glyphosate and AMPA residues in water

Standards stock, solvents and derivatization solutions – A standard stock solution (100 mg L^{-1}) of glyphosate and AMPA was prepared by dissolving 10 mg of each compound in a solution of Milli-Q water-acetonitrile (75 mL and 25 mL, respectively). This standard stock solution was diluted in Milli-Q water to obtain calibration solutions ranging from 0.01 mg L^{-1} to 0.20 mg L^{-1} required to set up the quantification method and obtain a standard curve. The borate buffer was obtained by dissolving 3.10 g of H_3BO_3, 3.75 g of KCl and 0.80 g of NaOH in 100 mL Milli-Q water. The pH was adjusted to 9 with NaOH 1 M. FMOC-Cl solution was prepared on the day of analysis by dissolving 10 mg of FMOC-Cl powder in 50 mL ACN. For the phosphate buffer 10 mM, used as eluent, 1.152 g of H_3PO_4 were dissolved in 1 L Milli-Q water and the pH was adjusted to 7 with NaOH 50%. Fifty mg of NaN_3 was added to the phosphate buffer to prevent contamination. The solution was filtered through a 0.22 µm pore membrane.

Sample preparation – Two mL of the water in which roots had been immersed was filtered through a 0.45 µm pore Nylon Acrodisc® to avoid clogging the HPLC column. One mL of each filtered sample was transferred to 2 mL vials (VWR Chemicals, Avantor). One vial per sample was prepared and each one was analyzed two times. For the derivatization, 200 µl of FMOC-Cl solution and 100 µl of borate buffer pH 9 solution were added to each vial and homogenized. Before HPLC analysis, the vials were left for 45 min to allow the derivatization reaction. The same protocol was used for calibration solutions, with one vial for each concentration and two analyses per vial.

HPLC-FLD analysis – Glyphosate and AMPA residues in water were quantified by high-performance liquid chromatography (HPLC) (1200 series, Agilent Technologies Santa Clara, CA, USA). To detect compounds, the HPLC system was coupled with a fluorescence detector (FLD) with excitation and emission wavelengths of 260 nm and 310 nm, respectively. The system also was equipped with an injection system and a quaternary pump. All the system was controlled by Agilent software. The analysis was performed with a Merck Lichrocart column C18 150 × 4.6 mm, with 5 µm particles, heated at 35 °C. The injection volume of samples was 10 µl. Solvents for the mobile phase consisted of 10 mM phosphate buffer (A) and 45:45:10 ACN:MeOH pH 7: Milli-Q water solution (B). The flow rate was 1 mL min^{-1}. The elution gradient was defined as follows: 0–1 min, 25% B; 10–15 min, 100% B; 17–22 min, 25% B. Peaks corresponding to glyphosate and AMPA were identified by comparing their retention times (RT) with chromatograms of calibration solutions.

For the quantification of glyphosate and AMPA in roots, different methods, adapted from ISO 21458, ISO 16308, Salingros (2020) and Liao et al. (2018), were tested. Unfortunately, glyphosate could not be detected due to assessment difficulties, the method is therefore not described here.

Exp. 3: Impact of glyphosate on spore germination and germ tube length

Experimental setup

According to the glyphosate concentration (i.e., 120.13 µg L^{-1}) detected in water in Exp. 2, six glyphosate concentrations were tested: 0, 12.01, 120.13, 1201.30, 2402.60 and 12,013 µg active ingredient per L water. These concentrations corresponded to 0, 0.1, 1, 10, 20 and 100 times the detected concentration. Each glyphosate solution was prepared by dissolving glyphosate powder (Sigma-Aldrich, Merck KGaA, Germany) in 5 mL of sterilized water. Each glyphosate solution (5 mL) was then added to 1 L sterilized MSR medium – with vitamins, solidified with 3 g L^{-1} Phytagel™ (Sigma-Aldrich, Merck KGaA, Germany) and adjusted to pH 5.5 before sterilization (121 °C for 15 min) – before being poured into each well of 24-well microplates (VWR International, Belgium). For the control treatment, only water was added.

Spores of *R. intraradices* MUCL 49410 were extracted from the hyphal compartment of a bi-compartmented Petri plate and individualized with needles. Healthy-looking spores were then placed individually in each well of the 24-well microplates. For each treatment, 36 replicates (i.e., spores) were considered. Microplates were incubated at 25 °C in the dark.

Evaluation of spore germination and germ tube length

Spore germination and germ tube length were monitored twice a week during 46 days under a stereomicroscope (10 to 35 × magnification). A spore was considered germinated as

soon as a germ tube emerged through the subtending hypha. The germ tube length was measured following the gridline intersect method adapted from Declerck et al. (2004). Briefly, a 1 mm × 1 mm grid was placed under each well. Each spore was observed and each intersection between a line and hyphae was recorded. Then, hyphal length was calculated by integrating the total number of intersections in the formula of Newman (Newman 1966):

$$L = \frac{\pi NA}{2H}$$

here L is the hyphal length, N is the intersection number, A is the gridline surface and H is the gridline length sum.

Statistical analysis

Data on spore germination rates, for the different concentrations of glyphosate and at each monitoring time, were analyzed using a Chi-square test. Data on germ tube length, at different time points, were analyzed using a Linear General Model (GLM) followed by Dunnett-HSD test to identify significant differences versus the Control treatment. Spores that did not germinate have not been considered for the mean length of germ tube. Normal distribution of residuals variance and normality were checked before analysis. When the conditions were not verified, a square root transformation was applied to the non-normal data.

Results

Exp. 1: Impact of mechanical or chemical (with contact or systemic herbicides) defoliation on the spore production dynamics of *R. irregularis* and *R. intraradices* associated with *S. tuberosum* or *M. truncatula*

Sporulation dynamics

Spore production dynamics are presented in Fig. 1. The first daughter spores were observed 3 weeks after plant-fungus association. The number of spores of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 increased gradually and weakly in both plants in the defoliation treatments. In contrast, the controls showed an

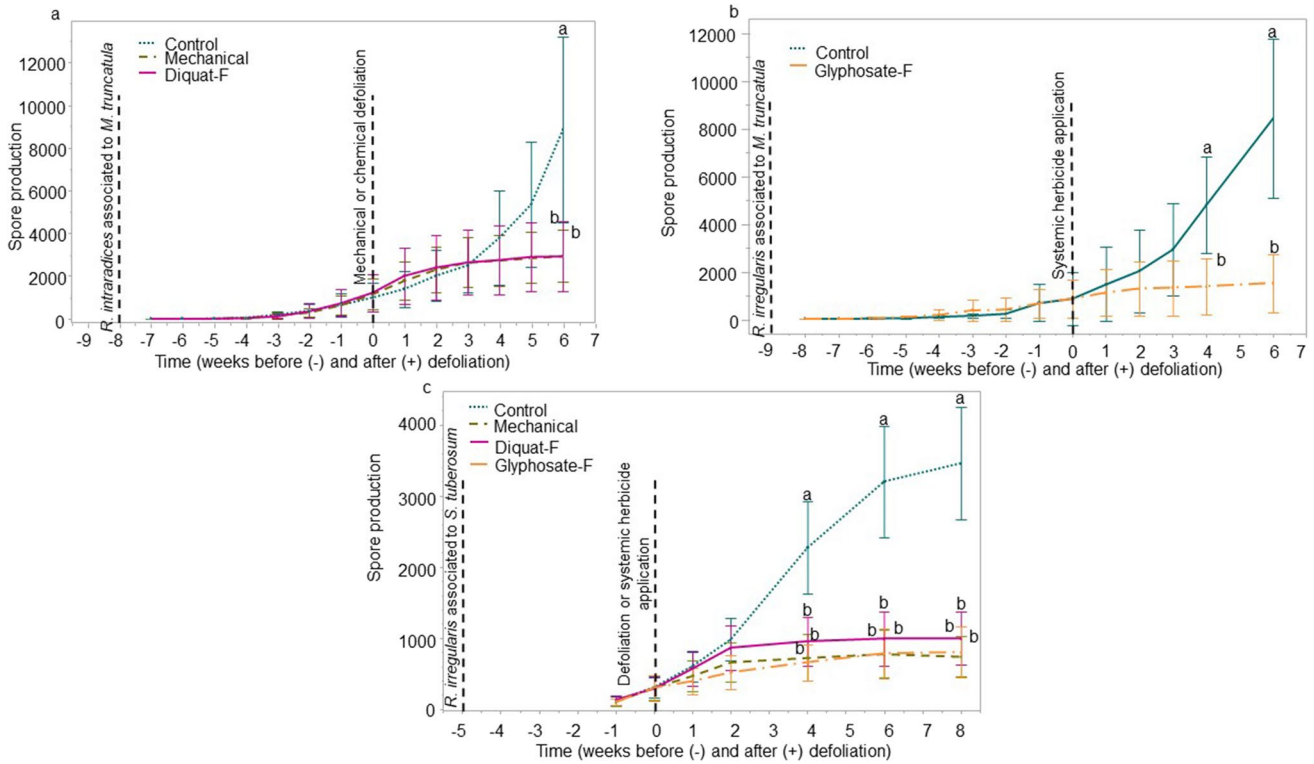

Fig. 1 Spore production dynamics of (**a**) *Rhizophagus intraradices* MUCL 49410 associated with in vitro grown *Medicago truncatula*, (**b**) *Rhizophagus irregularis* MUCL 41833 associated with in vitro grown *Medicago truncatula* and (**c**) *Rhizophagus irregularis* MUCL 41833 associated with in vitro grown *Solanum tuberosum*. Control plants were not defoliated. The other plants were defoliated mechanically (Mechanical) or chemically with a systemic herbicide (Glyphosate-F) or a contact herbicide (Diquat-F). Data are means ± 95% confidence limits (5 to 11 replicates (Table 1)). Means with the same letter were not statistically different at that week (HSD Tukey post-hoc test, $p < 0.05$)

exponential increase in the number of spores. A clear and general decrease in spore production rate, as quantified by the average weekly spore production rate from defoliation (R_t), was observed after plant defoliation (Fig. 2). The magnitude of the effect differed between defoliation practices and these differences changed as time passed after defoliation (Fig. 2). Overall, the results were quite similar for potato and barrel medic. Glyphosate-F had the strongest and fastest effect, decreasing weekly spore production rate already by 40% and 19% after 1 week, for potato and barrel medic, respectively. Mechanical defoliation and defoliation with Diquat-F affected spore production nearly as severely from six weeks after defoliation but at a slower and more progressive pace. For potato, mechanical defoliation significantly affected spore production from the first week, faster than defoliation with Diquat-F; at week 4, the impact of both defoliations became similar. For barrel medic, both mechanical defoliation and defoliation with Diquat-F affected spore production from week 2 only, and their effects never significantly differed until the end of the experiment (Fig. 2). Conclusions were robust to statistical imbalance (different number of replicated systems): the tests of the treatment, week and treatment-by-week effects, and the contrasts comparing the four defoliations on a week per week basis, were similar when tested on randomly down-sampled balanced datasets (with 5 replicates per defoliation level) (Table 3).

Root dry weight was assessed 8 and 6 weeks after defoliation of potato and barrel medic, respectively (Table 2). Whatever the practice, defoliation had a clear negative impact on root dry weight and colonization, which was similarly observed for potato and barrel medic (Table 2). With potato, no significant difference between the three defoliation treatments was observed. Conversely, with barrel medic, the negative effect of Glyphosate-F on dry weight and spores/vesicles percentage was significantly greater than after mechanical defoliation or defoliation with Diquat-F. Similarly for barrel medic, the negative effect of Glyphosate-F or mechanical defoliation on the arbuscules percentages was significantly greater than that of Diquat-F defoliation (Table 2).

Exp. 2: Analysis of glyphosate and AMPA residues in culture medium of *Solanum tuberosum* after glyphosate application

Glyphosate and AMPA were not detected in the non-treated controls. Glyphosate residues, but not AMPA, were detected in the water of glyphosate-F treated potato plants. The average concentration of glyphosate found in water samples was 120.13 ± 39.72 µg L^{-1} (Online Resource 3). This residue represents 0.11% of the applied amount of glyphosate on each plant.

Exp. 3: Impact of glyphosate on spore germination and germ tube length

The impact of glyphosate on spore germination and germ tube length was monitored for 46 days (at 6, 8, 12, 15, 18, 21, 25, 29, 32, 35, 39, 43 and 46 days). The spores of *R. intraradices* MUCL 49410 germinated after 4 days of incubation at 25 °C. The rate of germination, all treatments combined, ranged from 51.4% and 77.4% and this remained unchanged until the end of the experiment (Online Resource 4). None of the five concentrations of glyphosate had any impact on spore germination rate after 46 days ($\chi^2_5 = 6.307$; $p = 0.2775$) and there was no discernible difference over time.

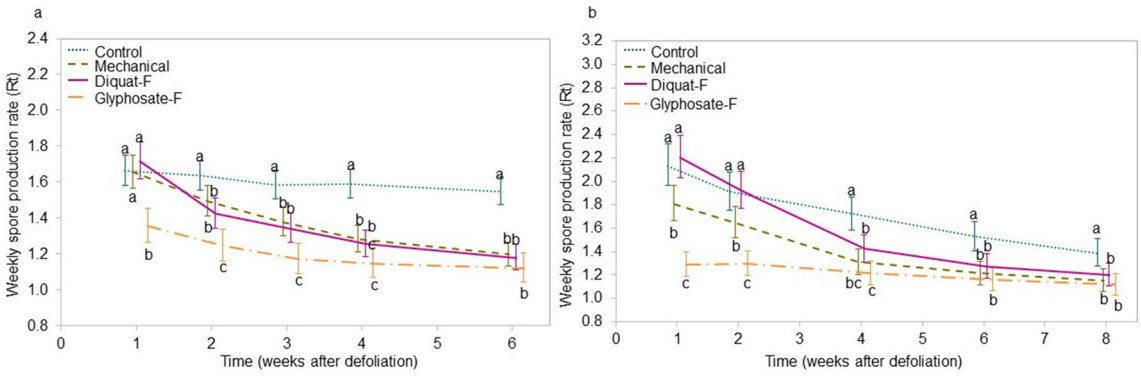

Fig. 2 Weekly rate of spore production (Rt) of Rhizophagus irregularis MUCL 41833 or Rhizophagus intraradices MUCL 49410 associated with in vitro grown (**a**) M. truncatula and (**b**) S. tuberosum, after defoliation treatment applied at week 0. Rt was computed as the average rate over the period from week 0 to week t; it is, therefore, a cumulative measure. Data are least-squares means ± 95% confidence limits. Means with the same letter were not statistically different at that week (3-way ANOVA with contrasts, see text for details). Points were slightly staggered for easier visualization. Table 3. Statistical analysis of average weekly spore production rates (Rt) using a three-way ANOVA (time and defoliation as fixed crossed factors, replicate as a random factor crossed with time but nested within defoliation)

Table 3 Statistical analysis of average weekly spore production rates (Rt) using a three-way ANOVA (time and defoliation as fixed crossed factors, replicate as a random factor crossed with time but nested within defoliation)

Source	Degrees of freedom	Sum of squares	Mean square	F-test	*P*-Value	
Potato						
Time	4	5.2767	1.3192	68.18	**< 0.0001**	
Defoliation	3	3.6325	1.2108	7.56	**0.0004**	
Replicate (Defoliation)	40	6.4092	0.1602	NA	NA	
Time × Defoliation	12	1.0055	0.0838	4.33	**< 0.0001**	
Time × Replicate (Defoliation)	160	3.0959	0.0193	NA	NA	
Error	0	0	NA	NA	NA	
Corrected total	219	19.4197				
Barrel medic						**% significant**
Time	4	1.0275	0.2569	31.61	**< 0.0001**	100.00
Defoliation	3	1.5982	0.53274	4.39	**0.0122**	60.00
Replicate (Defoliation)	27	3.2751	0.1213	NA	NA	
Time × Defoliation	12	0.2805	0.0234	2.88	**0.0018**	61.20
Time × Replicate (Defoliation)	108	0.8778	0.0081	NA	NA	
Error	0	0	NA	NA	NA	
Corrected total	154	7.0040				

"NA" denotes statistics that could not be computed given the ANOVA model used. Tests that revealed significant differences at the 0.05 α level are indicated in bold. "% significant": proportion of the 1000 perfectly balanced datasets with 5 replicates per defoliation group (produced from randomly down-sampled data) for which the effect was significant at the 0.05 α level, confirming that the results of the full analysis were affected only slightly by statistical imbalance of the original data. *Root weight and colonization by AMF*

The dynamics of germ tube growth were similar for all the treatments compared to the control (Online Resource 5). From day 6 to 46, the germ tube length at highest rate of glyphosate (100×) was less than that of all the other treatments but did not differ significantly from the control (Online Resource 6). From day 6 to 8, at a concentration of 120.13 µg a. i. L^{-1} (1×) of glyphosate, the germ tube length was significantly longer as compared to the control (Fig. 3). During the entire experiment – except at days 6, 18, 21, 29, 35 and 46 –, the lowest concentration of glyphosate (0.1×) significantly increased the germ tube length as compared to the control (Fig. 3). After 12 days, the germ tube length was numerically but not statistically longer for all treatments except 12,013 µg a. i. L^{-1} (100×) as compared to the control (Online Resource 6).

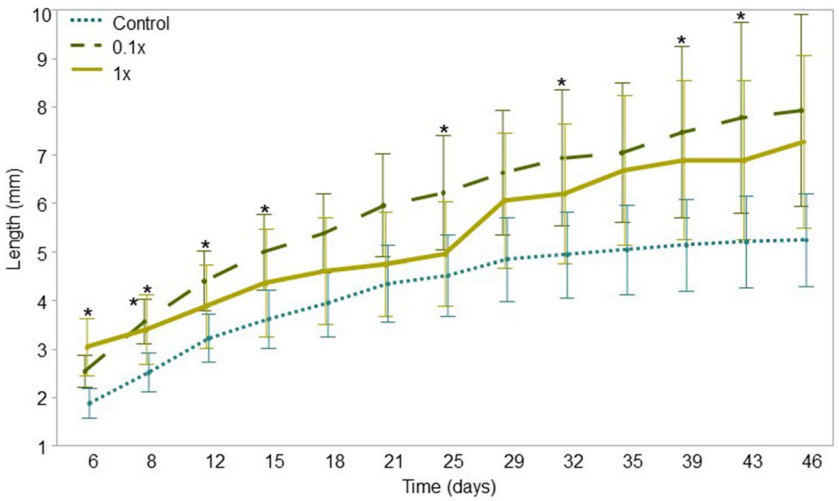

Fig. 3 Germ tube length of *Rhizophagus intraradices* MUCL 49410 for 46 days for Control (0 µg L^{-1}), 0.1×(12.01 µg L^{-1}) and 1×(120.13 µg $L.^{-1}$) treatments. The corresponding factor 1× is equal to the concentration of glyphosate detected in water in Experiment 2. Data are means ± 95% confidence limits of an average of 23 replicates. Means with * were statistically different from the control treatment at that day (Dunnett-HSD test, $p < 0.05$)

Discussion

Mechanical or chemical defoliation with a contact or systemic herbicide are likely to impact AMF. However, it is unknown whether the systemic herbicide (i.e., glyphosate) has a faster and stronger effect than the contact herbicide (i.e., diquat) or the mechanical defoliation, because the former affects all the plant parts, potentially impacting the AMF indirectly (by killing the plant) or directly (by accumulating in the roots and affecting the intraradical structures of the AMF or by release into the environment from decaying roots affecting the fungus in the soil). To explore this, we used a reproducible in vitro culture system and compared the impact of the three defoliation treatments on the sporulation dynamics of *R. irregularis* and *R. intraradices* associated with potato or barrel medic.

Whatever the defoliation practice, a clear and general decrease in the spore production rate of *R. irregularis* and *R. intraradices* was observed. This corroborates the study of IJdo et al. (2010), which suggested that the production of new spores depends directly on the C flow from the plant to the fungus. However, our study also revealed a difference between the three defoliation treatments. As hypothesized, glyphosate (the systemic herbicide) had a faster and stronger effect on spore production than the mechanical defoliation or the contact herbicide. Smid and Hiller (1981) demonstrated that glyphosate is translocated to potato roots 12 h after application. After 4 days, the quantity of glyphosate accumulated in roots has reached its maximum level (Smid and Hiller 1981). This translocation of glyphosate to the roots could directly interrupt the C flow from the roots to the AMF. It also may disrupt the functioning of the symbiosis because glyphosate inhibits EPSPS, an enzyme required in the shikimate pathway (Carlisle and Trevors 1988). This pathway is required to produce proteins involved in the exchange of signals between the host plant and AMF (Siqueira et al. 1991; Sheng et al. 2012). Finally, glyphosate may be released into the environment from decaying roots or root exudates, potentially directly affecting AMF spores (Coupland and Caseley 1979). From our results, it appears also that mechanical defoliation and the contact herbicide diquat have a slower and less pronounced impact on AMF spore production. Both defoliation practices inhibit plant photosynthetic production (by eliminating or drying the foliar part of the plant) but do not directly impact the roots. Because C present in the roots still could be translocated to the AMF, the impact of these defoliation practices was presumably slower and less pronounced than that of the systemic herbicide. However, the contact herbicide and mechanical defoliation affected spore production nearly as severely as the systemic herbicide six weeks after defoliation.

At harvest (i.e., 13 weeks after association of potato with *R. irregularis* MUCL 41833 or 15 or 14 weeks after association of barrel medic with *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410, respectively), mean root colonization for the control plants (i.e., those without defoliation) was relatively high. Conversely, defoliation practice, especially with glyphosate, reduced colonization by *R. irregularis* and *R. intraradices* and root dry weight. Druille et al. (2013b) also observed a significant reduction of root colonization (the number of arbuscules) after glyphosate application. They explained that arbuscules were sensitive to the reduction in the supply of carbohydrates caused by the herbicide. The contact herbicide diquat in contrast does not move to the roots. The impact of defoliation on arbuscules or intraradical spores/vesicles formation is thus probably only caused by photosynthesis disruption in the shoots and reduction/interruption of carbon flow to the roots. The impact of mechanical defoliation on arbuscules or intraradical spores/vesicles is comparable to that of the contact defoliant because only the foliar part of the plant is affected.

As suggested, the stronger and faster impact of glyphosate on spore production may result from the disruption of C metabolism in plants combined with the translocation of glyphosate from leaves to roots and from roots to soil. Translocation of glyphosate by plants is widely documented. Convincing results on root accumulation were mostly obtained with ^{14}C. For example, Coupland and Caseley (1979) in an experiment conducted in the greenhouse with *Agropyron repens*, noticed that 16% of the [^{14}C]glyphosate applied on the leaves was detected in the roots 8 days after the treatment. A similar quantity (i.e., 12%) was obtained by Laitinen et al. (2007) in quinoa roots after 8 days. Nevertheless, other studies showed that only 1 to 6% of [^{14}C]glyphosate applied to the shoot was found in roots after 24 h, depending on the glyphosate formulation (Duke 2021).

Exudation of glyphosate by roots also is studied widely. Coupland and Caseley (1979), noticed that 3.10% of applied glyphosate on quackgrass was exuded into the root-bathing solution. In another study conducted with field horsetail in a greenhouse, Coupland and Peabody (1981), showed that 0.36% of glyphosate applied on the aboveground plant parts was exuded in the root-bathing solution 4 days after application. Finally, Sesin et al. (2021) observed that 14 days after application of 2.20 mL of glyphosate (27 g L^{-1}) on *Ammannia robusta*, 1.22% of glyphosate applied was detected in the soil. These examples clearly demonstrate the exudation of glyphosate by roots and also highlight that the quantity of glyphosate translocated and exuded is dependent on the time of analysis and different parameters such as plant species and growth stage, soil type, temperature, dosage and experimental conditions (Smid and Hiller 1981; Masiunas and Weller 1988; Laitinen et al. 2007, 2009; Gomes et al. 2014; Singh et al. 2020).

Translocation of glyphosate into the roots is an important process which can impact AMF. However, glyphosate could not be detected in roots in our study. Glyphosate and AMPA

have chemical properties – low molecular weight and volatility, high polar character and water solubility and lack of chromophore or fluorophore – which makes extractions challenging (Kaczyński and Łozowicka 2015; Koskinen et al. 2016). The numerous studies found in the literature show the complexity of glyphosate quantification, which is dependent on the matrix and the organic matter content of the sample (Botero-Coy et al. 2013; Marek and Koskinen 2014; Koskinen et al. 2016). Plant material is a complex matrix in which endogenous unknown compounds interfere and react with FMOC-Cl (Koskinen et al. 2016; Tong et al. 2017). Low concentration levels also are an obstacle for precise quantification of glyphosate and AMPA residues (Liao et al. 2018).

In our study, only 0.11% of glyphosate applied on potato shoot and leaves was detected in the culture medium after 13 days. No other study, to the best of our knowledge, reported exudation of glyphosate by potato roots. For instance, Masiunas and Weller (1988) reported the absence of glyphosate in the growth medium of potato following leaf application. They supposed that the quantity was too low to be quantified. Although less frequent, some plant species, such as *Digitaria insularis*, did not exudate glyphosate (Barros et al. 2022). As AMPA is a secondary metabolite of glyphosate, its presence in samples also was assessed in our study. Unsurprisingly, no traces of AMPA were detected in the samples. In fact, AMPA is the major degradation product of glyphosate. Some microorganisms or genetically modified plants, as suggested by a study on glyphosate-resistant soybean (Sprankle et al. 1975; Reddy et al. 2004; Sviridov et al. 2015), have the capacity to degrade glyphosate into AMPA. Krzyśko-Lupicka et al. (1997) demonstrated that some fungal strains can degrade glyphosate into AMPA.

Overall, the low quantity of glyphosate detected in the water samples of potato suggested that glyphosate moved towards the roots, accumulating in these plant parts. There is thus a presumption of a direct effect of glyphosate on the intraradical structures of the AMF.

Finally, spore germination was tested in a dose–response approach using the concentration of glyphosate released in the water (i.e., 120.13 µg L^{-1}) to detect whether glyphosate had a direct effect on spore germination. We noticed a significant increase of germ tube length for concentrations 10 times lower or equal (only the first 2 days) than that exuded and no effect at higher doses (ranging from 1201.30 to 12,013 µg L^{-1}). To our knowledge, this increase has never been reported for glyphosate affecting AMF. This phenomenon is known as hormesis (a two-phased dose–response relationship, whereby a low concentration of a compound had a stimulatory effect on hyphal growth and a high concentration an inhibitory effect) and has been observed with other pesticides such as fenpropimorph (Rodriguez-Morelos et al. 2021). No fungicidal effect of glyphosate was observed on AMF spore development even at the high concentrations – slightly lower than the recommended field dose. This is corroborated by some studies, which showed that the growth of fungi was only inhibited at concentrations higher than the recommended field dose (Morjan et al. 2002; Dill et al. 2010). For example, under in vitro conditions, Giovannetti et al. (2006) showed that, for concentrations ranging from 0 to 72,800 µg L^{-1}, there is no impact on spore germination of *Funneliformis mosseae*, although an inhibition effect on germ tube growth was noticed at concentrations ranging from 4400 to 72,800 µg L^{-1}. Interestingly, Malty et al. (2006) demonstrated that the inhibitory effects of glyphosate on spore germination and germ tube growth is species and dose dependent. The higher the dose, the greater the inhibitory effect. The inhibitory germination and growth effect of glyphosate was slower and weaker for the *Gigasporaceae* family such as *Gigaspora margarita* and *Scutellospora heterogama* compared to the *Glomeraceae* family (*Glomus etunicatum*) which might be linked to the different life history strategies of AMF (Malty et al. 2006).

Conclusion

In this study we showed that mechanical/chemical defoliation strongly impact the dynamics of spore production of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 and root colonization of potato and barrel medic. The effect is more rapid and marked with the systemic herbicide (glyphosate) compared with the other two treatments (defoliation by mechanical treatment and contact herbicide) but fades with time. This difference in the short term is possibly attributed to the translocation of the active ingredient in the roots, directly impacting the transfer of C from root to AMF and possibly impacting also AMF present in the root. To the contrary, a direct effect of glyphosate (at the doses released from roots) released from decaying roots on spore viability and germ tube growth seems unlikely.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Statements and Declarations The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Competing interests The authors declare no competing interests.

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