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RESEARCH

Development of a high-throughput spore germination test to assess the toxicity of pesticides on arbuscular mycorrhizal fungi

Marjan Roshanfekrrad[1,2] · Christos Papadopoulos[4] · Maryline Calonne-Salmon[1] · Carolin Schneider[2] · Kunyang Zhang[3,5] · Dimitrios Karpouzas[4] · Stephan Declerck[1]

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Abstract

Pesticides are essential agricultural inputs that help securing crop yields. However, they can affect non-target soil microorganisms, including arbuscular mycorrhizal (AM) fungi, that are potential indicators of the toxicity of pesticides on the soil microbiota. Here, we developed a fast-track high-throughput spore germination test, for AM fungi produced in vitro. This test allows the determination of EC_{50} values and the nature of the effects of pesticides on AM fungal spores (fungicidal or fungistatic). First, 19 active ingredients were tested on *Rhizophagus intraradices* MUCL 49410. Secondly, five of these compounds, varying in their toxicity to *R. intraradices*, were tested on three additional AM fungi (*Rhizophagus irregularis* MUCL 41833, *Rhizophagus clarus* MUCL 46238 and *Rhizophagus aggregatus* MUCL 49408). Our results showed that the toxicity of pesticides varied according to their chemical nature, concentration and AM fungal species tested. With the exception of 3,5,6-trichloro-2-pyridinol (TCP, a transformation product of chlorpyrifos), insecticides and herbicides had no detrimental effect on spore germination at the concentration expected in soil upon application of the recommended dose, unlike most fungicides, which had an impact on one or more AM fungi. Fludioxonil and pyraclostrobin were by far the most problematic fungicide and *R. aggregatus* the most sensitive strain to pesticides. This AM fungus could thus be a good indicator to be used in standard ecotoxicity testing. In conclusion, we present a fast-track, high-throughput testing system for assessing the toxicity of pesticides on AM fungi, using spore germination as a relevant endpoint, that could be used as a first-tier screening tool in pesticide risk assessment.

Keywords Fungicide · Herbicide · Insecticide · EC50 value · Fungicidal · Fungistatic

Introduction

Pesticides are key agricultural inputs that help to protect crops from weeds, pests and diseases. At the same time, they can severely affect non-target plants and animals (Pagano et al. 2023) and impact composition, diversity and functions of microbial communities (Sim et al. 2022). This is why the European Commission has imposed a strict regulatory regime for the marketing authorization of pesticides by assessing their toxicity on various organisms from different trophic levels as indicators of environment quality. To date, however, microorganisms have not received the attention they deserve at regulatory level. It was only recently that the European Food Safety Authority (EFSA) identified certain soil microbial groups as indicators for monitoring the environmental risks of pesticides (Ockleford et al. 2017), and highlighted the need for new tests to assess the toxicity of pesticides on soil microorganisms (Karpouzas et al. 2022).

✉ Stephan Declerck
stephan.declerck@uclouvain.be

1 Universite Catholique de Louvain, Earth and Life Institute, Croix du Sud 2, Box L7.05.06, Mycology, Louvain-La-Neuve 1348, Belgium

2 INOQ GmbH, Solkau 2, 29465 Schnega, Germany

3 Department of Environmental Chemistry, Ueberlandstrasse 133, Eawag, Dübendorf 8600, Switzerland

4 Department of Biochemistry and Biotechnology, Laboratory of Plant and Environmental Biotechnology, University of Thessaly, Viopolis, Larissa 41500, Greece

5 Department of Chemistry, University of Zürich, Winterthurerstrasse 190, Zürich 8057, Switzerland

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One of the most important groups of soil microorganisms is the arbuscular mycorrhizal (AM) fungi. These are typical non-target microorganisms, present in most if not all agricultural soils, and obligatorily associated with the vast majority of economically important crops likely to be exposed to plant protection products (PPPs) (Sweeney et al. 2022). They perform important functions in the ecosystem such as (i) facilitate water and nutrients uptake by plants, (ii) improve soil structure and (iii) protect crops from infestations by pests and diseases (Gianinazzi et al. 2010). The application of pesticides to control, for example, pathogenic fungi or weeds in crops can affect AM fungi and, as a corollary, influence ecosystem services associated with them.

Fungicides are by far the most widely studied group of pesticides in terms of their effects on AM fungi, which are either direct (via contact between the pesticide and AM fungus) or indirect (via impact on the physiology of the host plant) (Calonne et al. 2012; Ren et al. 2021; Edlinger et al. 2022). Of the many studies carried out to date, a number have been conducted in vitro to examine the effects of fungicides on spore germination, hyphal length, root colonization, hyphal healing and anastomosis mechanisms. Spore germination and germ tube length, root colonization and extraradical mycelium growth of the widely used AM fungus *Rhizophagus irregularis* were negatively impacted by the addition of azoxystrobin, flutolanil, propiconazole, fenpropimorph and fenhexamid in the culture medium (Campagnac et al. 2008; Zocco et al. 2008; Calonne et al. 2010, 2012; Buysens et al. 2015). More recently, Rodriguez-Morelos et al. (2021, 2023) investigated the impact of azoxystrobin, fenpropimorph, flutolanil and pencycuron applied at 0.02 and 2 mg/L on the hyphal healing mechanism and the anastomonis formation per length of hyphae and noticed that azoxystrobin was the most detrimental at the highest concentration for both a *Gigaspora* sp. and *R. irregularis* species, while fenpropimorph impacted only *R. irregularis* (stimulating at low and inhibiting at high concentration). Conversely, flutolanil and pencycuron did not impact any of the two AM fungi. Uptake of phosphorus and transport to the host plant was also impacted by the application of fungicides (Zocco et al. 2011; Jakobsen et al. 2021). In field, more controversial results were obtained, with decrease or stimulation of root colonization as well as shifts in the AM fungal community, according to the class of fungicide, the tested molecule and its concentration and/or the considered tolerant/sensitive AM fungal specie (Ipsilantis et al. 2012; Jin et al. 2013; Rivera-Becerril et al. 2017).

Less attention has been paid to the impact of herbicides and insecticides on AM fungi, even though they account for 47.5% and 29.5% of the global pesticide market respectively (Pathak et al. 2022), and their potential off-target effects on microbial communities and functions in soil are acknowledged (Ipsilantis et al. 2012; Ruuskanen et al. 2023). This may be linked to the largely indirect effects of herbicides and the probable lack of effects of insecticides. For example, the negative impacts of the herbicide glyphosate on AM fungal root colonization and arbuscules density have been repeatedly observed (Giovannetti et al. 2006; Druille et al. 2013; Zaller et al. 2014; Helander et al. 2018). Karpouzas et al. (2014) also reported a negative impact of nicosulfuron, a field-applied sulfonylurea herbicide, on the abundance and diversity of AM fungi, although they could not conclude if the effect was direct (toxicity on the AM fungus) or indirect (toxicity on the plant). Silva et al. (2014) noted a reduction of root colonization of *Eucalyptus* spp. in presence of a combination of the herbicides sulfentrazone, oxyfluorfen, and isoxaflutole. Regarding insecticides, Hamel and Strullu (2006) did not report any negative effects on AM fungi. This was later contradicted by Wang et al. (2011) reporting that phoxim inhibits AM fungal colonization of *Daucus carota* but not *Allium fistulosum*. Similarly, Ipsilantis et al. (2012) reported that azadirachtin (a triterpenoid insecticide) inhibited the growth of *Claroideoglomus etunicatum* and caused a significant shift in the AM fungal community.

Whatever the pesticide, it is necessary to evaluate its effects on AM fungi as key soil microorganisms. However, there are over 1000 pesticides used worldwide, each with different properties and toxicological effects. It is therefore necessary to develop standardized methods for assessing the effects of pesticide active ingredients (a.i.) on the non-target microbial group of AM fungi.

The life cycle of AM fungi often, but not exclusively, begins with the germination of a spore and the emission of a germ tube growing toward a host root. Spores are thus key propagules for initiating the symbiosis and any direct effect of PPPs on spore germination can result in failure of root colonization. The measurement of the direct effect of active ingredient (a.i.) on AM fungal spores could reflect their exposure to pesticide residues in soil, although it should be kept in mind that other factors (e.g., soil pH, soil organic matter content, temperature and soil moisture) may modulate pesticide persistence and microbial community composition, and hence their impacts on AM fungi. That said, it remains essential to evaluate the effects of the a.i. of pesticides comparing toxicity endpoint values using a standard, easy-to-use and reproducible method.

Here we designed a fast-track, high-throughput in vitro cultivation system based on microwell plates that enables the assessment of the toxicity of increasing concentrations of PPPs on AM fungi. Nineteen a.i. of pesticides widely used in Europe (6 fungicides, 7 herbicides, 6 insecticides), with different mode of action, were tested through this system for toxicity on spore germination of the model AM

fungus *Rhizophagus intraradices*. We extended the study to three other *Rhizophagus* species (*R. irregularis*, *Rhizophagus clarus* and *Rhizophagus aggregatus*) and tested their response to five of the initial 19 a.i. of pesticides. These five pesticides were selected according to their toxicity towards *R. intraradices*, spanned from non-toxic to highly toxic, hence they would allow us to get a view of differences in the sensitivity of different AM fungi to pesticides.

Materials and methods

AM fungal strains

Four AM fungal strains were obtained from the Glomeromycota in vitro collection (GINCO – http://www.mycorrhiza.be/ginco-bel): *R. intraradices* MUCL 49410, *R. irregularis* MUCL 41833, *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408. They were selected for their ability to grow in vitro and for their production of thousands of spores in a short period of time, necessary to achieve the germination assays. They were maintained in vitro in bi-compartmented Petri plates (St-Arnaud et al. 1996) on the Modified Strullu-Romand (MSR) medium (Declerck et al. 1998) until thousands of spores were produced.

Active ingredients

Nineteen pesticide a.i. (6 fungicides, 6 insecticides and 7 herbicides), with purity ≥ 98%, varying in their mode of action and mode of application were selected (Table 1 and details of the products in Supplementary Data). The effects of pesticide a.i. on AM fungi was assessed at five concentrations (0.01x, 0.1x, 1x, 10x and 20x, with 1x being the recommended dose for agricultural use in Europe – see Table 1) as explained below. Because some pesticides could have long half-life and thus accumulate in soils after several applications, we tested 10 and 20x the recommended dose for a thorough analysis of the potential impact of pesticides on the AM fungal germination. The assumptions used for the calculation of the concentrations tested are provided in supplementary material and were based on relevant EFSA documents (EFSA2015a, b).

With the exception of glyphosate and its transformation product, aminomethyl phosphonic acid (AMPA), which are soluble in water, all the other a.i. tested were hydrophobic. Therefore, they were first dissolved in acetone before being

Table 1 List of selected active ingredient ("1x" is the concentration that corresponds to the maximum recommended dose rate used in Europe and calculated as described in supplementary Data)

Pesticides Common Name	Type of pesticide	Concentration 1x (mg/L)	Mode of action
Acetamiprid	Insecticide	1.02	Interrupts the transmission of nerve impulses by antagonizing the nicotinic acetylcholine receptor
Acrinathrin	Insecticide	0.4	Blocks the operation of Na/K channels
Chlorpyrifos	Insecticide	15.7	Blocks the activity of acetylcholinesterase in the central nerve system of insects
Pyrethrum	Insecticide	1.21	Blocks the operation of Na/K channels
Spiromesifen	Insecticide	3.77	Inhibits acetyl-CoA-carboxylase, a lipid metabolism enzyme
3,5,6-Trichloro-2-pyridinol (TCP)	Chlorpyrifos transformation product	3.46	Unknown
Clethodim	Herbicide	2.35	Inhibits acetyl-CoA-carboxylase, a lipid metabolism enzyme
Clopyralid	Herbicide	2.77	Mimicks plant growth hormone auxin, and when administered at effective doses, cause uncontrolled and disorganized plant growth that leads to plant death
Glyphosate	Herbicide	21.6	Inhibits enolpyruvylshikimate-3-phosphate synthase in the shikimic acid pathway, preventing the biosynthesis of aromatic amino acids
Metribuzin	Herbicide	5.38	Inhibits photosynthesis by disrupting electron transfer in photosystem II
Metsulfuron-methyl	Herbicide	0.42	Inhibits acetolactate synthase involved in synthesis of branched chain amino acids
Tembotrione	Herbicide	1.54	Inhibits hydroxylphenyl pyruvate dioxygenase, leading to inhibition of carotene pigment synthesis
Aminomethyl phosphonic acid	Glyphosate transformation product	21.6	Unknown
Etridiazole	Fungicide	9.6	Inhibits ergosterol biosynthesis
Fludioxonil	Fungicide	3.85	Inhibits transport-associated phosphorylation of glucose
Flutolanil	Fungicide	32.77	Inhibits mitogen-activated protein-kinase
Hymexazol	Fungicide	0.56	Inhibits synthesis of DNA/RNA
Iprodione	Fungicide	12.12	Inhibits mitogen-activated protein-kinase
Pyraclostrobin	Fungicide	3.08	Blocks the electron transport at the outer side of the cytochrome-bc1 complex

added into the MSR medium, lacking sucrose and vitamins and solidified with 3 g/L Phytagel™. Glyphosate and AMPA were directly incorporated in the MSR medium. Two controls were further considered, one with water (for glyphosate and AMPA) and one with acetone (5 ml/L) (Zocco et al. 2008) added to the MSR medium (for the other a.i).

Design of a fast-track high-throughput in vitro system to assess the toxicity of pesticides on AM fungal spore germination

The impact of a.i. of pesticides on spore germination was evaluated in 12 wells-microplates (Thermo Fisher Scientific Inc., Waltham, US). Thirty-six spores (i.e., replicates) distributed at random in 3 microplates (corresponding to 12 spores per microplate), were considered per a.i. and concentration. Two ml of MSR medium lacking sucrose and vitamins and supplemented or not with the a.i. before solidification with 3 g/L Phytagel™ were added in each well. Spores of each AM fungal strain were collected from the hyphal compartment of the bi-compartmented Petri plates with fine needles and tweezers. They were transferred to MSR liquid medium lacking sucrose and vitamins and gently detached from the hyphae with needles to obtain isolated spores. The spores were examined under a binocular microscope and those that were empty or injured were discarded. Healthy-looking spores (i.e., characterized by the presence of numerous lipid droplets) were then transferred individually with a micropipette to each well and were incubated at 27 °C in the dark.

Spore germination

Spore germination was assessed at 1, 3, 7, 10, 15, 21 and 28-days post-inoculation under binocular microscope at 45X magnification. In the first assay, the spore germination of *R. intraradices* MUCL 49410 in the presence of the 19 pesticide a.i. was tested. Based on the EC_{50} values obtained (see below), 5 a.i. (TCP, glyphosate, etridiazole, hymexazol and pyraclostrobin) were further tested in a second assay for their effects on spore germination of three other AM fungal strains (*R. irregularis* MUCL 41833, *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408) plus *R. intraradices* (as the internal standard to validate the results of the first assay) to identify potential differences in the sensitivity of the different AM fungal strains to pesticides. As described in Mallman et al. (2018), we defined a minimum germination rate of 75% after 28 days of growth in the acetone and water control to validate the assay. In our study, the lowest percentage of germination measured in both controls was 97.2, 80.6, 94.4 and 86.1 for *R. intraradices*, *R. irregularis*, *R. clarus* and *R. aggregatus*, respectively.

Toxicity endpoints values (EC_{50})

In our study, EC50 values describes the concentration of the pesticide that reduces half of the germination at the last day of the spore production dynamics (i.e., 28 days). The calculation of EC_{50} values was performed according to Papadopoulou et al. (2020).

Determination of the fungicidal or fungistatic effect of the pesticides

To determine the fungicidal or fungistatic effect (Chiocchio et al. 2000; Zocco et al. 2008; Buysens et al. 2015) of each concentration of the a.i., only treatments for which more than 50% of the spores did not germinate were considered. Non-germinated spores were transferred to new microplates containing pesticide-free MSR medium lacking sucrose and vitamins and solidified with 3 g/L Phytagel™. Spore germination was assessed over a period of one month and pesticides a.i. classified into five categories: totally fungicidal if 0% of spores germinated, strongly fungicidal if the % of spores germination ranged between 1 and 40%, intermediately toxic if the % of spores germination ranged between 41 and 60% (i.e., between fungicidal and fungistatic), strongly fungistatic and totally fungistatic if the % of spores germination ranged between 61 and 99% or was 100%, respectively (the schematical representation of the scale is presented on Figs. 4 and 5).

Statistical analysis

The spores germination in presence of the different pesticides and the different concentrations were only compared to their respective water or acetone control, using the fit generalized estimating equations model (GEE), considering time-points as repeated measures, using the package "geepack" (Halekoh et al. 2006). Dose-response curves "*drc*" (v3.0-1) package (Ritz and Streibig 2005) of the R software (R Core Team 2017) were used for the analysis of EC_{50}. A detailed description of the tested models can be found in Ritz et al. (2015). The "*drc*" package provides an extensive suite of tools for non-linear regression of dose-response relationships. The process of selecting the most appropriate model was guided by an empirical evaluation of goodness-of-fit indices, employing maximum likelihood estimation for parameter determination, followed by the choice of the three-parameters log-logistic model, for which the lower limit is equal to 0 (LL.3), which was the best compromise among tested models for comparing endpoint values. The statistical analyses were done with RStudio 4.1.1 (Packages: "*readxl*" (v1.4.3), "*xlsx*" (v0.6.5), "*forcats*" (v1.0.0), "*geepack*" (v1.3.12), "*multcomp*" (v1.4-25) and "*drc*" (v3.0-1)).

Results

Spore germination of *Rhizophagus intraradices* MUCL 49410 is affected by the type of pesticides

The impact of insecticides (Fig. 1), herbicides (Fig. 2) and fungicides (Fig. 3), at five concentrations was evaluated on spore germination of *R. intraradices* MUCL 49410 during 28 days. No significant differences were observed between the two controls (i.e., water and acetone) (see Fig S1 in supplementary material). The first germinations were already observed after one day and reached between 60 and 100% within a week. After 28 days, nearly 100% of the spores in both controls had germinated. Therefore, in the statistical analysis below, the water control was used as control for glyphosate and AMPA treatments and the acetone control for the other a.i.

Effects of insecticides on spore germination (Fig. 1)

At 0.01x and 0.1x the recommended pesticide dose (RPD)[1], the % spore germination varied between 72% and 100% after 28 days of incubation, whatever the a.i. With the exception of acrinathrin at 0.01x and 0.1x the RPD, pyrethrum and spiromesifen at 0.01x the RPD, all the a.i. showed a significantly lower % of spore germination. This significant difference is explained by a lower % of spore germination the first days of the dynamics in comparison with the control, which is not visible at the end of the dynamic, for all a.i., except chlorpyrifos (around 74% of germination for both concentrations).

At 1x the RPD and above, no effect was measured in presence of acrinathrin. On the other hand, a significantly lower % of spore germination was noticed for all the a.i. at these concentrations except for pyrethrum and TCP at 1x and spiromesifen at 20x the RPD. As for the low concentrations, the % of spore germination was lower the first days of germination but reached the control at the end of the dynamic in presence of acetamiprid, pyrethrum and spiromesifen (between 80 and 100% of germination) while chlorpyrifos and TCP remained below the control at the end of the dynamic (74% for chlorpyrifos and 44 and 0% for TCP at 10 and 20x the RPD, respectively).

Effects of herbicides on spore germination (Fig. 2)

At 0.01x and 0.1x the RPD, only glyphosate and AMPA showed no significant effect in comparison with the control. A significantly lower % of spore germination was noticed for clethodim, clopyralid and tembotrione at these two concentrations, and 0.1x for metsulfuron-methyl and metribuzin. This significant difference is explained by a lower % of spore germination the first days of the dynamics in comparison with the control, which is not visible at the end, for all the a.i. (between 90 and 100% of germination at 28 days), except for clethodim (around 75% of germination).

At 1x the RPD and above, metribuzin and AMPA showed no significant effect at any of these three concentrations. On the other hand, the % of spore germination was significantly lower in presence of clethodim, clopyralid, metsulfuron-methyl and tembotrione and in presence of glyphosate at 10x and 20x the RPD. This effect was visible the first days of the germination dynamics but the % was similar to the control at the end of the dynamic for clopyralid at 1 and 10x the RPD, metsulfuron-methyl and tembotrione for all concentrations and glyphosate at 10x the RPD (between 80 and 100% of germination). At 28 days, % of spore germination remained lower in presence of 1, 10 and 20x the RPD of clethodim and reached 72, 53 and 39%, respectively. No germination was observed in presence of glyphosate at 20x the RPD.

Effects of fungicides on spore germination (Fig. 3)

At 0.01 and 0.1x the RPD, a significantly lower % of spore germination was observed for all tested fungicides except for hymexazol at 0.1x the RPD and iprodione at both concentrations. The effect was visible on the graphs the first days of the germination dynamic whereas the germination was similar to the control at the end of the germination dynamic (between 90 and 100% of germination) for both concentrations of etridiazole and hymexazol and for pyraclostrobin at 0.01x the RPD. In presence of both concentration of flutolanil and 0.1x the RPD of pyraclostrobin, the % of spore germination was comprised between 70 and 80%, while for fludioxonil no germination was observed.

At 1x the RPD and above, the % of spore germination was significantly lower for all the tested a.i., except for hymexazol at 20x the RPD. This effect was not visible for hymexazol at 10x the RPD and etridiazole at 1x the RPD at the end of the dynamic (around 90% of spore germination). To the contrary, the % of spore germination remained low in presence of 1x the RPD of iprodione and pyraclostrobin (25 and 11% respectively). With etridiazole at 10 and 20x the RPD, spores started to germinate after 15 days and reached around 55% of germination at the end of the dynamics, as in presence of the three tested concentrations of flutolanil. No germination was observed in presence of iprodione and pyraclostrobin at 10 and 20x the RPD and in presence of fludioxonil at all concentrations.

[1] Refer to the concentration expected in soil upon application of the recommended dose.

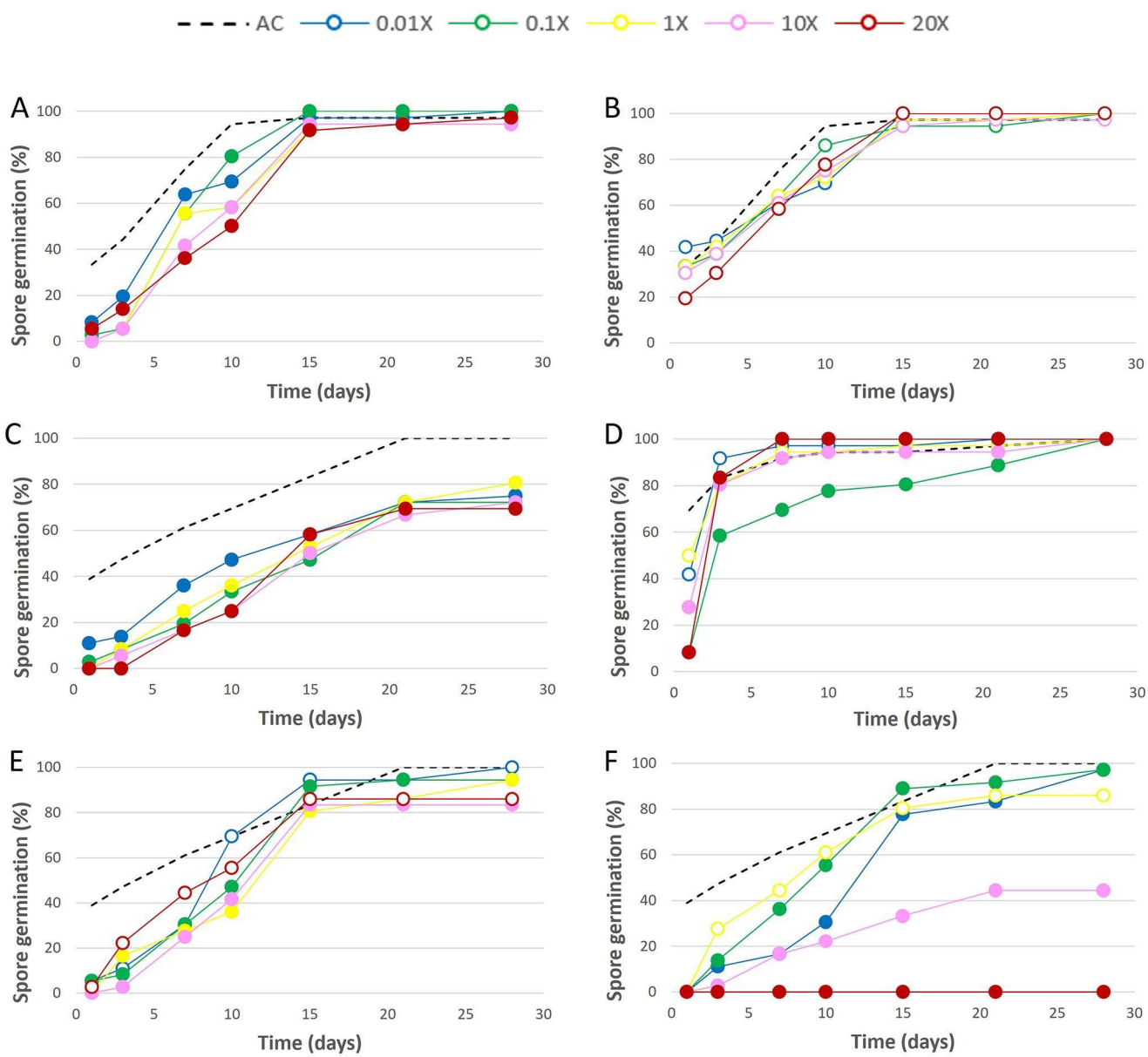

Fig. 1 Percentage of spore germination (%) of *R. intraradices* MUCL 49410 in the presence of the active ingredients of the insecticides. (**A**) acetamiprid, (**B**) acrinathrin, (**C**) chlorpyrifos, (**D**) pyrethrum, (**E**) spiromesifen and (**F**) TCP (the transformation product of chlorpyrifos), at 0.01x (blue), 0.1x (green), 1x (yellow), 10x (pink) and 20x (red), with 1x being the concentration expected upon application of the agricultural recommended dose rate. Closed circles show a significant difference with the control (small dash) whereas open symbols show no significant difference with the control, according to the fit generalized estimating equations model ($p \leq 0.05$; $n = 36$ spores). Each concentration of each molecule was compared with its respective control

AM fungal species differ in their sensitivity to selected pesticides

The impact of TCP, glyphosate, etridiazole, hymexazol and pyraclostrobin on the % of spore germination of *R. intraradices* (used in experiment 1) and on % spore germination of *R. irregularis*, *R. clarus* and *R. aggregatus* was evaluated during 28 days (Table 2 and Fig. S2, Fig. S3 and Fig. S4 of supplementary materials).

Effect of TCP

The % of spore germination was significantly lower for all tested fungal strains in comparison with their respective control at all tested concentrations except for *R. irregularis* at 0.1x the RPD and *R. intraradices* at 1x the RPD (Fig. S2). At 0.01x and 0.1x the RPD, the % of spore germination was above 75% for all the strains at day 28 (Table 2). Exception to this was *R. aggregatus*, which reached a % of spore

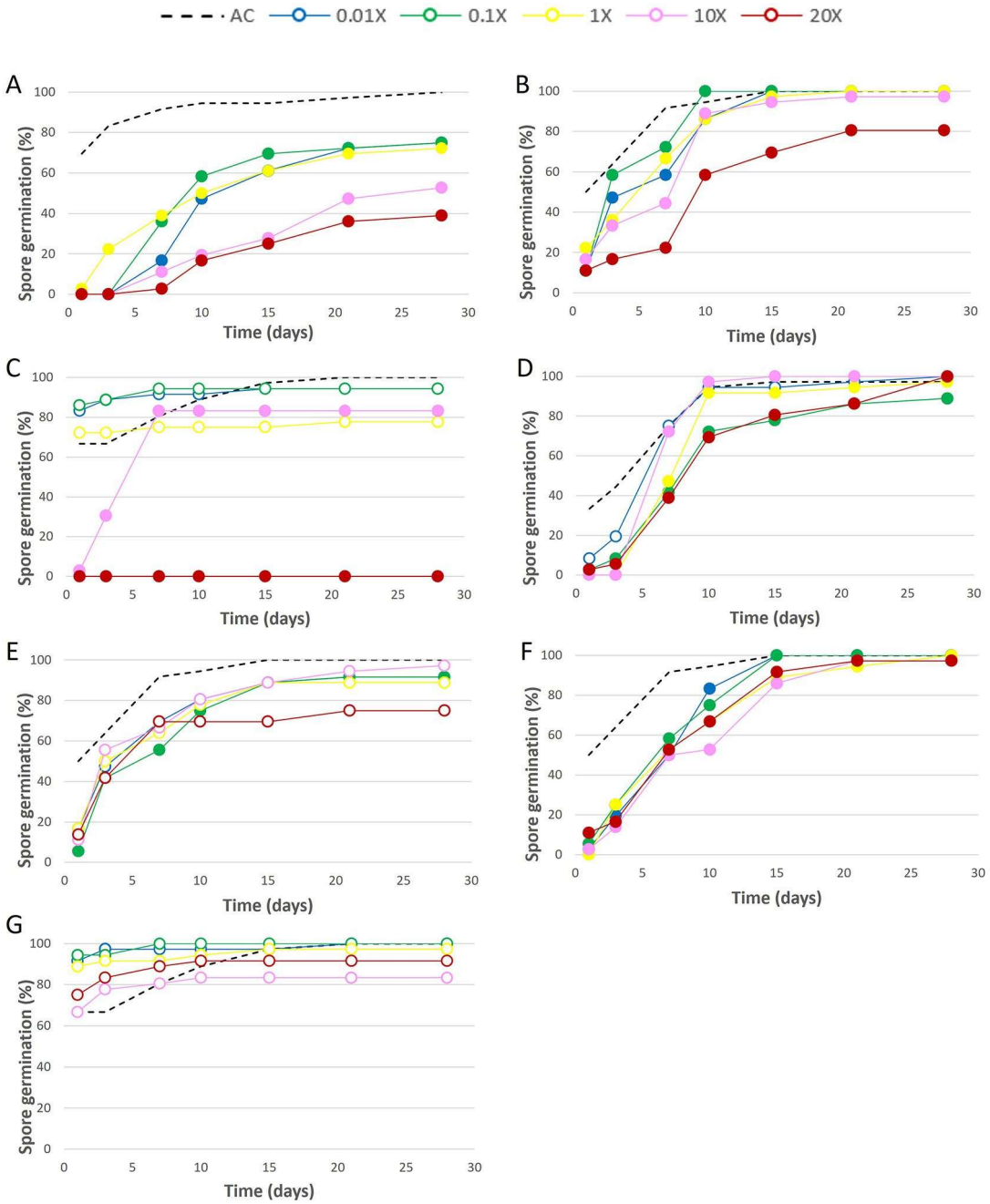

Fig. 2 Percentages of spore germination (%) of *R. intraradices* MUCL 49410 in the presence of the active ingredients of the herbicides. (**A**) clethodim (**B**) clopyralid, (**C**) glyphosate, (**D**) metsulfuron-methyl, (**E**) metribuzin, (**F**) tembotrione and (**G**) AMPA (the transformation product of glyphosate), at 0.01x (blue), 0.1x (green), 1x (yellow), 10x (pink) and 20x (red), with 1x being the concentration expected upon application of the agricultural recommended dose rate. Closed circles show a significant difference with the control (small dash) whereas open symbols show no significant difference with the control, according to the fit generalized estimating equations model ($p \leq 0.05$; $n = 36$ spores). Each concentration of each molecule was compared with its respective control. Acetone was the control for all molecules except for glyphosate and AMPA, which were compared with the water control

germination of 5.5% (Table 2 and Fig. S2), whereas its control reached 88%. At 1x the RPD, the % of spore germination of *R. aggregatus* and *R. irregularis* was almost zero (at day 28), while it reached 86% for *R. intraradices* and 100% for *R. clarus*. At 10x the RPD, *R. intraradices* was the only AM fungus showing germination (44% at day 28), whereas at 20x the RPD, no spore germination was noticed for any of the AM fungi.

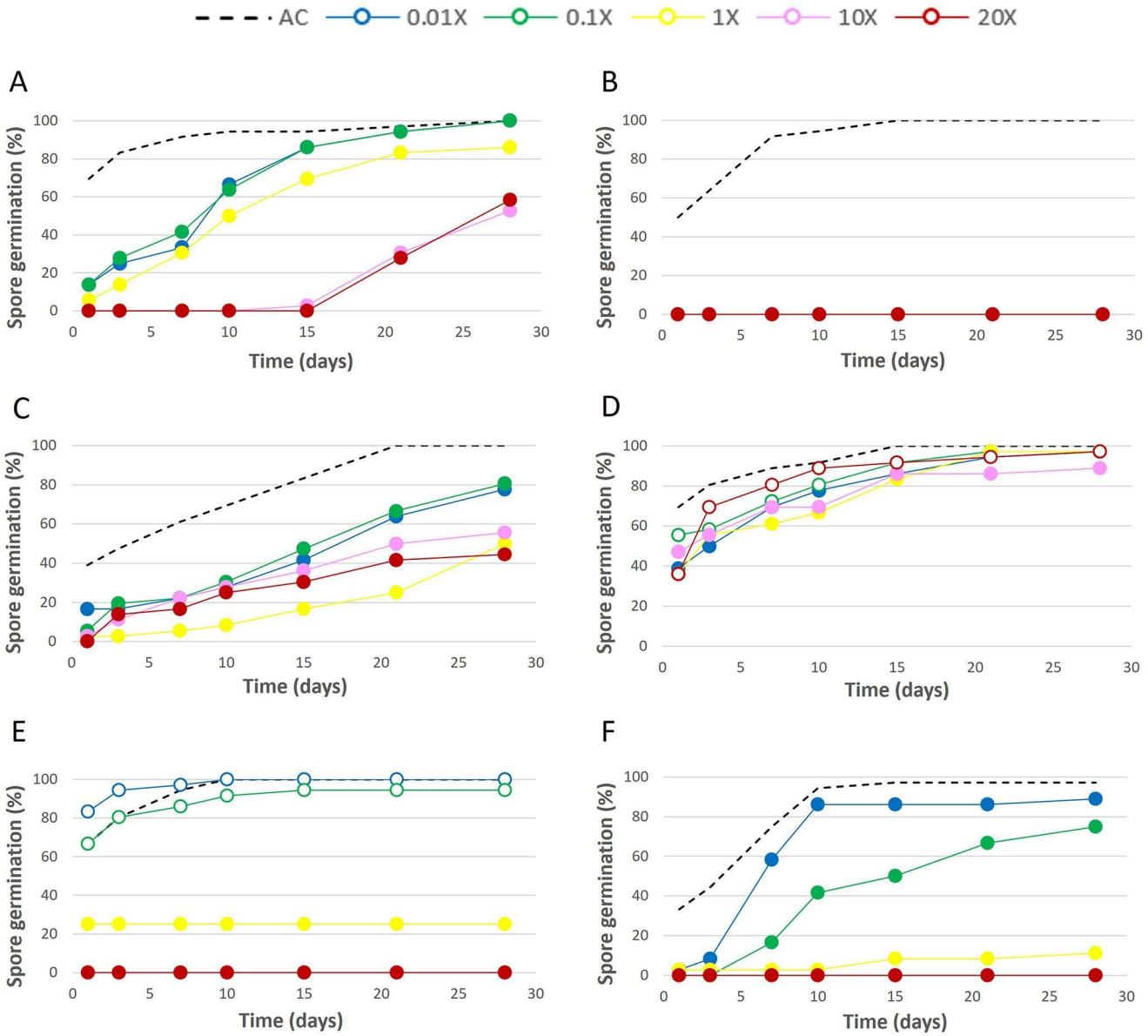

Fig. 3 Percentages of spore germination (%) of *R. intraradices* MUCL 49410 in the presence of the active ingredients of the fungicides. (**A**) etridiazole, (**B**) fludioxonil, (**C**) flutolanil, (**D**) hymexazol, (**E**) iprodione and (**F**) pyraclostrobin, at 0.01x (blue), 0.1x (green), 1x (yellow), 10x (pink) and 20x (red), with 1x being the concentration expected upon application of the agricutlural recommended dose rate. Closed circles show a significant difference with the control (small dash) whereas open symbols show no significant difference with the control, according to the fit generalized estimating equations model ($p \leq 0.05$; $n = 36$ spores). Each concentration of each molecule was compared with its respective control

Effects of glyphosate

At 0.01x and 0.1x the RPD, the % of spore germination of the AM fungi was above 83% at day 28 (Table 2). Whereas no significant differences with the control was observed for *R. intraradices*, *R. irregularis* and *R. aggregatus*, *R. clarus* presented a significant lower % of spore germination as compared to its control for both concentrations but the effect was not visible at the end of the dynamic (Fig. S3). At 1x the RPD, the spores of all AM fungal species germinated similarly to their control, except *R. aggregatus*, for which the % of spore germination was significantly lower as compared to the control but reached 91.7% at day 28. At 10 and 20x the RPD, glyphosate decreased the % of spore germination of all AM fungi. Only *R. clarus* and *R. intraradices* were able to germinate, reaching 94 and 83% of spore germination, respectively, whereas at 20x the RPD, only *R. clarus* germinated after one week, to reach 89% at the end of the experiment.

Table 2 Percentages of spore germination (%) of *R. intraradices* MUCL 49410, *R. irregularis* MUCL 41833, *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408 after 28 days of incubation in presence of the active ingredients of TCP, glyphosate, etridiazole, hymexazol and pyraclostrobin at 0.01x, 0.1x, 1x, 10x and 20x, with 1x being the concentration expected upon application of the agronomic recommended dose rate. $n = 36$ spores

Active ingredient	Concentration	*R. intraradices*	*R. irregularis*	*R. clarus*	*R. aggregatus*
TCP	0.01x	97.2	75	100	5.5
	0.1x	97.2	86.1	100	5.5
	1x	86.1	0	100	2.8
	10x	44.4	0	0	0
	20x	0	0	0	0
Glyphosate	0.01x	94.4	97.2	100	88.8
	0.1x	94.4	83.3	97.2	88.8
	1x	77.8	86.1	97.2	91.6
	10x	83.3	0	94.4	0
	20x	0	0	88.9	0
Etridiazole	0.01x	100	77.8	97.2	36.1
	0.1x	100	86.1	97.2	30.5
	1x	86.1	86.1	100	25
	10x	52.8	91.7	97.2	27.8
	20x	58.3	88.9	91.7	11.1
Hymexazol	0.01x	97.2	77.8	94.4	83.3
	0.1x	97.2	63.8	100	27.8
	1x	97.2	88.9	97.2	8.3
	10x	88.9	88.9	100	38.9
	20x	97.2	75	100	100
Pyraclostrobin	0.01x	88.9	97.2	69.4	0
	0.1x	75	19.4	0	0
	1x	11.1	0	0	0
	10x	0	0	0	0
	20x	0	0	0	0

Effects of etridiazole, hymexazol and pyraclostrobin

In presence of etridiazole, only the % of spore germination of *R. irregularis* was not significantly different compared to its related control at 0.01, 0.1 and 1x the RPD (Fig. S4). The % of spore germination of *R. aggregatus* did not exceed 36% even at 0.01x the RPD and remained significantly lower as compared to its control until the end of the experiment (Table 2 and Fig. S4). The % of spore germination of *R. intraradices* and *R. clarus* was significantly lower than the control at 0.01x, 0.1x and 1x the RPD but this effect was not visible at the end of the dynamics (above 85% of spore germination). The % of spore germination for *R. intraradices* at 10 and 20x the RPD were significantly lower than the control (less than 60% at day 28). At the same concentrations, *R. irregularis* and *R. clarus* showed a significantly lower % of spore germination, but they reached almost 90% of germination at day 28.

In presence of hymexazol, the % of spore germination of *R. intraradices* and *R. clarus* were similar to the control at the end of the germination dynamics (above 88.9%) while a lower germination rate was generally recoded the first days (Table 2 and Figure S5). On the other hand, % of spore germination remained lower than 80% for *R. irregularis* in presence of 0.01, 0.1 and 20x the RPD (78, 64 and 75% respectively), whereas it remained similar to the control at 1 and 10x the RPD. The % of spore germination of *R. aggregatus* was significantly lower, except for the concentration 0.01x the RPD. But the effect was not clearly demonstrated at 20x the RPD (100% of germination at 28 days), whereas the % of spore germination reached 27.8, 8.3 and 38.9% at 0.1, 1 and 10x the RPD, respectively, at 28 days. This observation could be due to a dissolution problem or a potential hormetic effect.

In presence of pyraclostrobin, all AM fungal strains had a % of spore germination above 69% at 0.01x the RPD, with the exception of *R. aggregatus*, which did not show any germination at all concentration tested (Table 2 and Fig. S6). At the same concentration, a significant reduction in the % of spore germination was observed for *R. intraradices* and *R. clarus*, even if *R. intraradices* presented a % of spore germination around 89% at the end of the dynamic (Fig. S6). At 0.1x the RPD, only *R. intraradices* and *R. irregularis* were able to germinate but their % of spore germination remained significantly lower (75 and 19.4%, respectively) as compared to their respective controls. At 1x the RPD, *R. intraradices* was the only AM fungus able to germinate but the % of spore germination was significantly lower in comparison with the control. At 10x and 20x the RPD, no spore germination was noticed for any of the AM fungal strains.

EC_{50} values of the pesticides tested on AM fungal strains

EC_{50} values of pesticides on R. intraradices

The mean EC_{50} values are reported in Table 3. For *R. intraradices*, the EC_{50} values for the fungicides ranged between <0.03 to 282.8 mg/L, while insecticides and herbicides were considered as safe against *R. intraradices*. Within the group of insecticides, TCP had a mean EC_{50} of 34.25 mg/L, which is almost 10 times the RPD and for the other tested insecticides, the mean EC_{50} values were higher than the highest tested concentration (i.e., >20x the RPD). Among herbicides, the mean EC_{50} value of glyphosate was 259.2 mg/L and for clethodim it was 29.8 mg/L, which were almost 12 times the RPD for both of them. Fungicides as etridiazole and hymexazol showed mean EC_{50} values of 192 and 11.2 mg/L respectively (>20x the RPD). Flutolanil had a mean EC_{50} of 282.8 mg/L which was 8.6 times the RPD.

Table 3 Mean effective concentration (EC_{50}, mg/L) of insecticides, herbicides and fungicides as derived from testing their effects on % spore germination for *R. intraradices* MUCL 49410, *R. irregularis* MUCL 41833, *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408. Data are presented as means ± standard error. The asterisk indicates that the mean EC_{50} value is below the minimum concentration tested and double asterisk indicates that the mean EC_{50} value is above the maximum concentration tested. The EC_{50} are categorized in four color groups, with inhibitory effect on AM fungi at concentrations (1) above 20x the RPD (dark green), (2) between 20x and the RPD (light green), between 0.01x and the RPD (light red) and below 0.01x the RPD (dark red)

		R. intraradices	*R. irregularis*	*R. clarus*	*R. aggregatus*
Pesticide Group	**Pesticide Active ingredient**	***mean EC_{50} (mg/L)***	***mean EC_{50} (mg/L)***	***mean EC_{50} (mg/L)***	***mean EC_{50} (mg/L)***
Insecticide	Acetamiprid	>20.4**			
	Acrinathrin	>8**			
	Chlorpyrifos	>314**			
	Pyrethrum	>24.2**			
	Spiromesifen	>75.4**			
	TCP	34.25 ± 0.56	1.28 ± 1.92	12.9 ± 4.9	< 0.03*
Herbicide	AMPA	>432**			
	Clethodim	29.8±8.42			
	Clopyralid	>55.4**			
	Glyphosate	259.2±11.2	40.6±3.33	>432**	75.6±6.31
	Methsulfuron methyl	>8.4**			
	Metribuzin	>107.6**			
	Tembotrione	>30.8**			
Fungicide	Etridiazole	>192**	>192**	>192**	< 0.096*
	Fludioxonil	<0.04*			
	Flutolanil	282.81 ± 7.12			
	Hymexazol	>11.2**	>11.2**	>11.2**	1.01 ± 91.1
	Iprodione	6.42 ± 0.05			
	Pyraclostrobin	0.8 ± 0.03	0.25 ± 0.09	0.039 ± 0.05	<0.03*

Iprodione and pyraclostrobin showed a mean EC_{50} value of 6.42 and 0.8 mg/L respectively, which were almost 0.5 and 0.3 times the RPD, respectively. Fludioxonil had a mean EC_{50} value which was lower than the lowest tested concentration (< 0.01x the RPD).

EC_{50} values of selected pesticides on other AM fungal strains

The EC_{50} values of the five selected pesticides on the three AM fungal strains are reported in Table 3. According to the EC_{50} values, *R. intraradices*, *R. clarus* and *R. irregularis* presented similar tolerance to pesticides, except for TCP. *R. irregularis* presented a lower EC_{50} value than *R. intraradices* and *R. clarus* for this pesticide (0.3x the RPD for *R. irregularis*). For *R. aggregatus*, the EC_{50} value was lower than the lowest concentration tested for TCP, etridiazole and pyraclostrobin, denoting a particular sensitivity of this strain to pesticides. Glyphosate showed a high mean EC_{50} value in all strains. Regarding etridiazole, EC_{50} value exceeded the highest tested concentration for all tested strains except for *R. aggregatus*, for which the EC_{50} value was lower than the lowest tested concentration. Similarly hymexazol had an EC_{50} value higher than the highest tested concentration (11.2 mg/L), which corresponds to >20x the recommended dose rate, for all strains tested except for *R. agreggatus*, for which an EC_{50} value of 1.01 mg/L was calculated corresponding to a concentration much lower than what is expected to have in soil after application of the 1x the RPD. Pyraclostrobin showed the most toxic effect against *R. aggregatus* with a mean EC_{50} value of < 0.03 mg/L which is much lower than the RPD. Likewise, for *R. intraradices*, *R. clarus* and *R. irregularis* it presented a mean EC_{50} value of 0.8, 0.039 and 0.25 mg/L, or between 0.01 and 1x the RPD.

Fungicidal/fungistatic effect of pesticides on AM fungal spore germination

Fungicidal/fungistatic effect of pesticides on R. intraradices

The spores that had not germinate after 28 days in presence of the a.i. of pesticides were transferred to a pesticide-free MSR medium and assessed for germination after one month (Figs. 4 and 5). Only the pesticides and concentrations inhibiting spore germination by at least 50% were taken into account. Among the insecticides, only the spores of *R. intraradices* grown in the presence of TCP at 10x and 20x the RPD were tested. At these concentrations, 85 and 60% of the spores transferred germinated, suggesting a strong fungistatic effect at 10x the RPD and an intermediate effect at 20x the RPD (Fig. 4). Among the herbicides, 92% of the spores initially grown in presence of 20x the

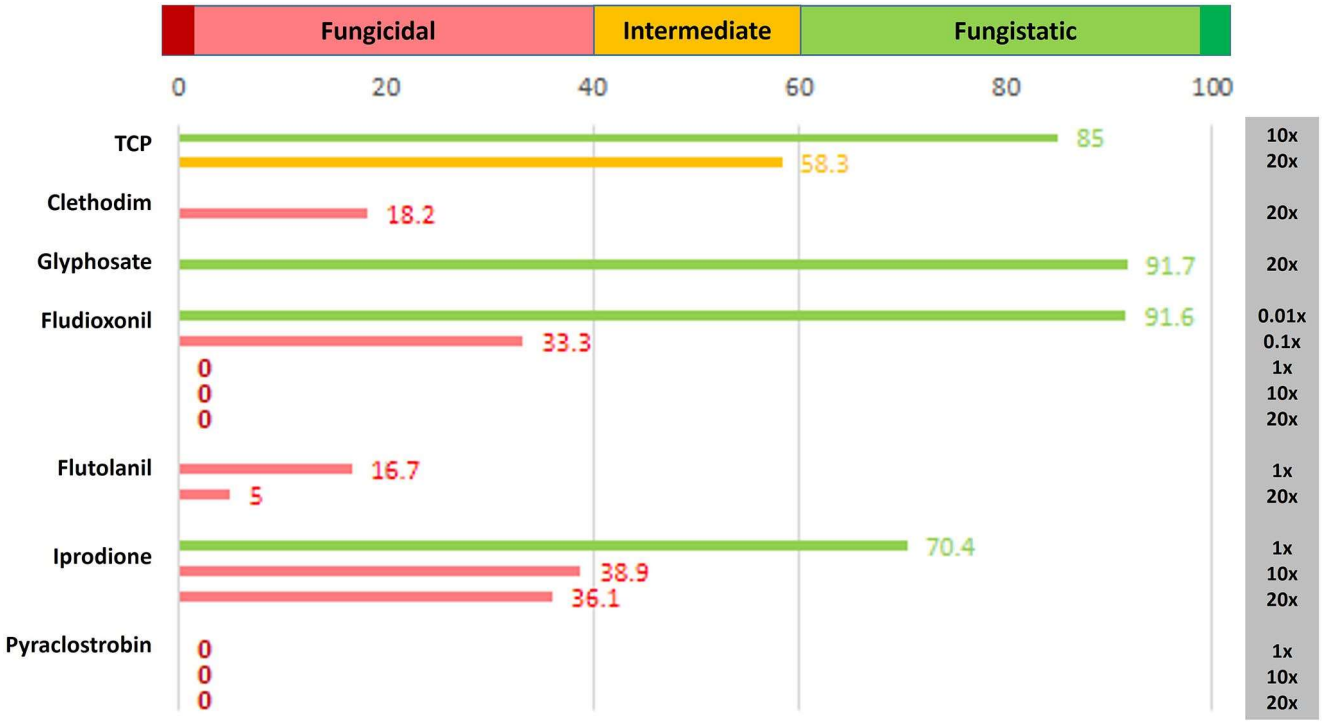

Fig. 4 Fungicidal or fungistatic effects of pesticides on spore germination of *R. intraradices* MUCL 49410. Spores germination was assessed one month after their transfer to a pesticide-free MSR medium. Only the pesticides and concentrations which inhibited the germination of at least 50% of the spores after 28 days were considered. In the absence of germination on pesticide-free medium, the pesticide was considered as totally fungicidal (0% spore germination - dark red), strongly fungicidal (1 to 40% spore germination - light red), intermediate (i.e., between fungicidal and fungistatic) (41 to 60% spore germination - orange), strongly fungistatic (61 to 99% spore germination - light green) and totally fungistatic (100% spore germination - dark green)

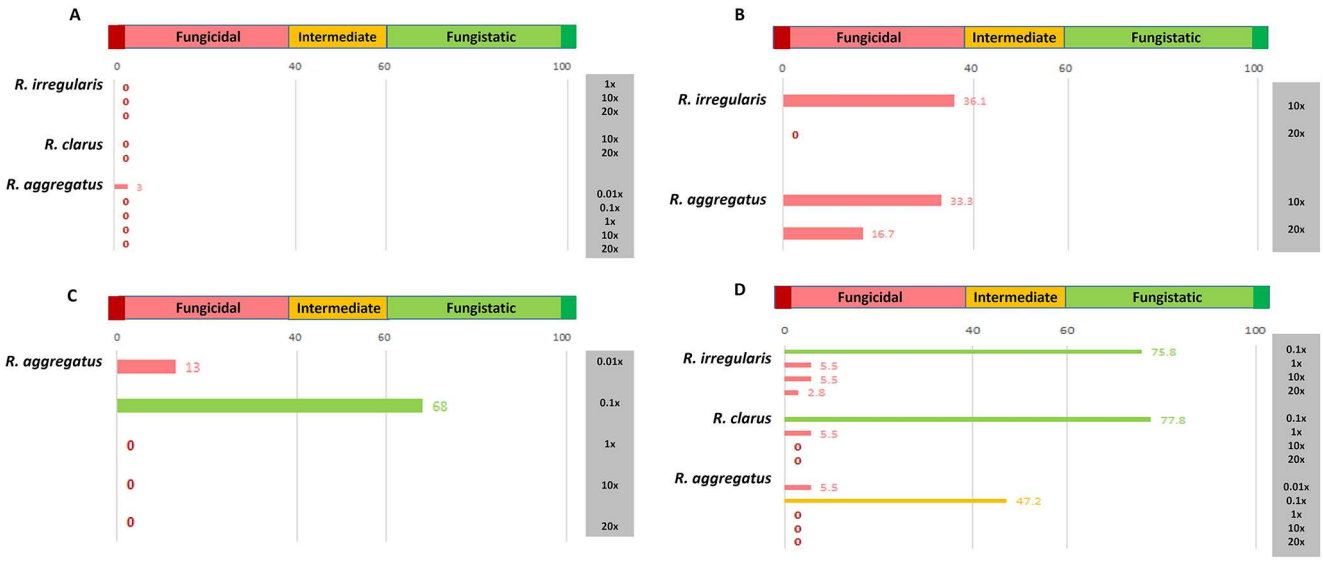

Fig. 5 Fungicidal or fungistatic effects of pesticides (**A**) TCP, (**B**) glyphosate, (**C**) etridiazole and (**D**) pyraclostrobin, on spore germination of *R. irregularis*, *R. clarus* and *R. aggregatus*. Spores germination was assessed one month after their transfer to a pesticide-free MSR medium. Only the pesticides and the concentrations which inhibited the germination of at least 50% of the spores after 28 days were considered. In the absence of germination on pesticide-free medium, the pesticide was considered as totally fungicidal (0% spore germination - dark red), strongly fungicidal (1 to 40% spore germination - light red), intermediate (i.e., between fungicidal and fungistatic) (41 to 60% spore germination - orange), strongly fungistatic (61 to 99% spore germination - light green) and totally fungistatic (100% spore germination - dark green)

RPD of glyphosate germinated after transfer on pesticide-free medium, suggesting a strong fungistatic effect. On the other hand, a strong fungicidal effect was observed for spores initially grown in the presence of clethodim at 20x the RPD, with only 18.2% of spores able to germinate after transfer to pesticide-free MSR medium (Fig. 4). Among the fungicides, only 33% of spores initially grown on fludioxonil at 0.1x RPD were able to germinate on the pesticide-free medium, suggesting a strong fungicidal effect. On the other hand, at concentrations above 1x RPD, no germination was observed, suggesting a total fungicidal effect. Similar observation was made for pyraclostrobin at the same concentrations. Regarding flutolanil, only a few spores initially grown at 1x and 20x the RPD (16.7% and 5%, respectively), were able to germinate after their transfer on flutolanil-free medium, suggesting a strong fungicidal effect. Iprodione at 1x the RPD had a strong fungistatic effect, with only 29.6% of spores not able to germinate after transfer on pesticide-free medium. On the other hand, at 10x and 20x the RPD, these a.i. had a strong fungicidal effect with only 38.9% and 36.1%, respectively, of spores able to germinate after their transfer on iprodione-free medium (Fig. 4).

Fungicidal/fungistatic effects on R. irregularis, R. clarus and R. aggregatus

A similar test was undertaken for the spores of *R. irregularis*, *R. clarus* and *R. aggregatus* that did not germinate in the presence of pesticides (Fig. 5). TCP was totally fungicidal for all tested species, except for *R. aggregatus* at 0.01x the RPD, for which 3% of the spores were able to germinate after their transfer on TCP-free medium, suggesting a strong fungicidal effect (Fig. 5A). Glyphosate had a strong fungicidal effect. At 10x the RPD, only 36.1% of the spores of *R. irregularis* germinated on the glyphosate-free medium and none after incubation at 20x the RPD. This percentage was also low for *R. aggregatus* with only 33.3% and 16.7% of the spores that germinated after their incubation in presence of glyphosate at 10x and 20x the RPD, respectively (Fig. 5B). Etridiazole at 0.01x the RPD had a strong fungicidal effect with only 13% of the spores of *R. aggregatus* able to germinate after transfer on the pesticide-free medium. Curiously at 0.1x the RPD the effect was strongly fungistatic with 68% of the spores able to germinate after transfer on etridiazole-free medium. At 1x the RPD and above, etridiazole was totally fungicidal to *R. aggregatus* (Fig. 5C). Pyraclostrobin at 0.1x the RPD had a strong fungistatic effect with 75.8% of the spores of *R. irregularis* able to germinate after transfer on pesticide-free medium (Fig. 5D). At 1x the RPD and above, pyraclostrobin showed strong fungicidal effect on *R. irregularis* with only a few spores (<6%) able to germinate after transfer on pyraclostrobin-free medium. A similar effect was observed with *R. clarus* with a strong fungistatic effect at 0.1x the RPD (77.8% of spores germination) and strong fungicidal effect at 1x the RPD (5.5% of spore germination) and total fungicidal effect above 1x the RPD (no germination). For *R. aggregatus*, and intermediate fungicidal effect was noticed at 0.1x the RPD with 47.2% of the spores able to germinate after transfer on pesticide-free medium, a strong fungicidal effect at 0.01x the RPD (55.5% of spore germination) and a total fungicidal effect at 1x the RPD and above (no spore germination) (Fig. 5D).

Discussion

Numerous studies have been carried out to test the toxicity of pesticides on AM fungi using in vitro and greenhouse experimental approaches, focusing on spore germination, root colonization and physiological parameters (Calonne et al. 2012; Rodriguez-Morelos et al. 2021; Edlinger et al. 2022). Although spores reflect only part of the AM fungal life cycle, they are good indicators for use in ecotoxicological tests for a range of reasons: (i) they are involved in the first phase of root colonization (ii) they are independent of the host plant, making it possible to distinguish direct effects on AM fungi from indirect effects caused by alterations to host plant physiology induced by pesticides (iii) they constitute the life stage of AM fungi that they are most vulnerable to exposure to pesticides in soil (Campagnac et al. 2008; Calonne et al. 2012; De Novais et al. 2019a, b). This has led to the implementation of an ISO 10832:2009-03 standard based on spore germination in a sandwich system placed in sand or artificial substrate, as a criterion for assessing toxicity of PPPs. Mallmann et al. (2018) demonstrated that this protocol was suitable to test the impact of three fungicides (i.e., chlorothalonil, mancozeb and metalaxyl-M) on spore germination of *Gigaspora albida* and *R. clarus*, two AM fungal species with different life history strategies.

An alternative to this protocol is the use of AM fungal spores produced in vitro on root organs (Cranenbrouck et al. 2005) and their evaluation under axenic conditions on a gelled synthetic growth medium amended with the targeted PPP. Although in vitro tests on spore germination should be considered as simplified tests of little ecological relevance, they are useful as a first-tier of ecotoxicological risk assessment (Sweeney et al. 2022). Spores produced in vitro have several advantages being (i) free of any unwanted microbial contaminant, (ii) easy to monitor non-destructively (iii) reproducible and adaptable to high-throughput assessment approaches involving screening of a large number of molecules. In this case, they can also serve as early indicators for the selection of chemistries that conform with

sustainable soil health during the discovery of novel pesticide compounds.

Here, we have developed a fast-track in vitro cultivation system based on the incubation of healthy-looking in vitro produced spores in microwells containing the a.i. of pesticides at increasing concentrations. Using this system, we have been able to test, in a dose-response scheme, the impact of 19 different pesticides commonly used in Europe on the germination of spores belonging to several AM fungal species grown in vitro. The system is also suitable for differentiating fungistatic and fungicidal effects and for calculating EC_{50} values that could be used in risk assessment. *Rhizophagus intraradices* MUCL 49410 was used in two independent experiments, giving the same results, demonstrating the reproducibility of the fast-track in vitro culture system developed and the suitability of this AM fungus for testing PPPs.

Based on the EC_{50} values, the pesticides tested can be categorized in three groups (1) pesticides showing an EC_{50} value higher than the RPD (1x), (2) pesticides with EC_{50} values lower than 1x the RPD, (3) pesticides with EC_{50} varying from below to above 1x the RPD depending on the AM fungi tested (see Table 3).

Effects of insecticides, herbicides and their transformation products on spore germination

With the exception of TCP, a transformation product of the insecticide chlorpyrifos, and the herbicides clethodim and glyphosate, all the insecticides and herbicides a.i. tested on *R. intraradices* MUCL 49410 had an EC50 exceeding 20x the RPD and were classified in Group 1. These a.i. only induced a delayed response of a few days, which was reversed on day 28. TCP and glyphosate had EC_{50} values lower than 10x the RPD and were thus tested on three other strains (*R. irregularis*, *R. clarus* and *R. aggregatus*). TCP had a moderate effect on *R. intraradices* and *R. clarus* and a strong effect on *R. irregularis* and *R. aggregatus* at concentrations below 1x the RPD and was thus classified in Group 3 but this effect was more fungistatic than fungicidal. Because of the long persistence of TCP in groundwater (half-life estimated between 44 and 77 years (Cheremisinoff and Rosenfeld 2011), a tolerance variation between the AM fungal species could be expected. Conversely, glyphosate did not affect % spore germination of any of the three other strains at 1x the RPD and EC_{50} values remained above 1x the RPD for all the strains, it could thus be classified in Group 1. In presence of this a.i., 70% of the spores of *R. intraradices* germinated within the 28 days of observation at 1x the RPD or below and the effect of this herbicide was totally fungistatic. Even if a significant impact of this molecule was measured on the AM fungal life steps and viability (Druille et al. 2016; Wilkes et al. 2020; Bastogne et al. 2024), the short half-life of this molecule in water (estimated around 10 days (Lewis et al. 2016) could explain the limited impact on the spore germination in the culture medium in the present work. This result confirms the report of Pasaribu et al. (2011) which demonstrated that glyphosate at concentrations up to the recommended field rate does not affect spore germination of *Funneliformis mosseae.* The direct inhibition of spore germination was observed only when glyphosate concentration levels exceeded the recommended field dosage. This is in agreement with our findings, where the application of glyphosate at concentrations higher than 1x the RPD affected the germination process in all strains except for *R. clarus*.

The results obtained in our study does not rule out the possibility that other insecticides or herbicides may affect spore germination as shown by Malfatti et al. (2023) with imidacloprid and thiamethoxam (two insecticides) tested at increasing concentration (10, 50, 100, 250, 500, 750 and 1000 mg a.i./kg) on *R. clarus* and *G. albida*. In their study, all tested concentrations of imidacloprid significantly reduced spore germination of both strains, while thiamethoxam affected spore germination of *G. albida* only at concentrations above 100 mg a.i./kg and at all concentrations on *R. clarus*. However, it is important to note that spore germination of both strains was recorded after only 14 days of incubation, and it cannot be ruled that longer periods (i.e. 28 days as in our study), could have mitigated this effect.

Effects of fungicides on spore germination

Among the tested fungicides, only flutolanil can be classified in Group 1 because it impacted the spore germination of *R. intraradices* only at concentrations above 1x the RPD. The limited impact of flutolanil on spore germination corroborates earlier studies by Buysens et al. (2015) under in vitro condition with *R. irregularis* MUCL 41833. Likewise, in the study of Rodriguez-Morelos et al. (2021), flutolanil did not affect the hyphal healing mechanism in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331. The absence of effect could not be linked to its half-life in water, because of the long persistence of flutolanil (half-life comprised between 90.5 and 400 days (Lewis et al. 2016; Yang et al. 2019)).

Conversely, fludioxonil, iprodione and pyraclostrobin inhibited spore germination at concentration below 1x the RPD and were thus classified in Group 2 exhibiting mostly fungicidal effect (except for iprodione with more fungistatic effect). Fludioxonil belongs to the phenylpyrrole class of chemistry and has a unique mode of action which prevents fungal respiration and reduces mycelial growth rate in a broad range of fungi (Wang et al. 2022). This fungicide

totally inhibited spore germination even at the lowest concentrations exhibiting fungistatic effects at 0.01x the RPD and fungicidal effect at 1x the RPD and above. Iprodione has half-life comprised between 149 days at pH5 to 3 days at pH7 (European Commission Health & Consumer Protection Directorate 2002). The possibility of the absence of effect on the target enzymes in AMF fungi, unlike in phytopathogenic fungi, could not be excluded. Pyraclostrobin belongs to the strobilurin class of chemistry and inhibits the fungus respiratory chain, being an excellent preventive and curative agent against a wide range of fungal pathogens (Dominguez et al. 2021). It totally inhibited the germination *R. aggregatus* spores and had inhibitory effect below 1x the RPD on *R. intraradices*, *R. clarus* and *R. irregularis*. This result corroborates with the study of Rodriguez-Morelos et al. (2021) who reported a strong inhibitory effect of azoxystrobin (another fungicides of the strobilurin group) at 2 mg/L on the extraradical network of *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331.

Hymexazol and etridiazole can be classified in Group 3 because of their diverse effects on the *Rhizophagus* strains tested. Both fungicides had a strong inhibitory effect (below the RPD) on *R. aggregatus* but not on the three other AM fungi tested (*R. intraradices*, *R. irregularis* and *R. clarus*). The half-life of these fungicides is relatively short (less than 8 days for hymexazole and from 7 to 98 days for etridiazole (US-EPA 2016; Lewis et al. 2016; Ewere et al. 2024). The spectrum of this fungicide is limited to Oomycetes and can thus be the reason of its low impact on three *Rhizophagus* strains. Interestingly, *R. aggregatus* was reported as a very sensitive AM fungus to Ag NPs (a nano-pesticide and fertilizer) (Abd-Alla et al. 2016). It thus might be considered as a good candidate for use in regulatory spore emergence tests with pesticides. In addition, *R. aggregatus* is an AM fungus found in numerous commercial AM inoculum. Their persistence in soil and capacity to colonize plants, and thus efficacy to stimulate plant growth and yield in conventional agriculture, is questionable.

Conclusion

Overall our study show that pesticides impact spore germination, with an effect varying according to the type of pesticide (insecticides, herbicides and fungicides), the concentration at which it is applied and the AM fungal species tested. With the exception of TCP (a transformation product of chlorpyrifos), the insecticides and herbicides tested had no effect on spore germination at the concentration expected in soil upon application of the recommended dose and their effects were more fungistatic than fungicidal. Conversely fungicides showed higher potency against AM fungi as expected with four out of the six a.i. tested showing considerable inhibitory effects on at least one AM fungal strain which were more fungicidal than fungistatic. The fungicides fludioxonil, iprodione and pyraclostrobin had a strong inhibitory impact on spore germination which suggest that other fungicides of the same chemical groups may also exhibit high toxicity on AM fungi and should be prioritized for testing. A fast track-high througput in vitro system, based on spore germination, was developed as a first-tier assessment of the ecotoxicity of pesticide on AM fungi. Still further tests (in vitro and in vivo) with a wider range of pesticides are required to verify their ecotoxicological relevance. Indeed, AM fungi in agricultural soil may have developed a certain tolerance to the applied pesticides. On the other hand, many other aspects like application method of pesticides in the soil, soil characteristics and microbial interactions may affect the persistence of these pesticides in the soil and finally their impact on AM fungi that these results can differ from those obtained in vitro. Despite its little ecological relevance due to the influence of the soil characteristics and the microbial activity, the present method can be used "*to filter out harmless substances that do not require further investigation under more realistic exposure scenarios in higher-tier testing frameworks*" as suggested by Sweeney et al. (2022).

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Author contributions MJ, MCS, CS and SD conceived and designed the study. MJ performed the experiments. KZ and MCS conducted the calculations of EC50. MJ. MCS, KZ, DK and SD analyzed the data. All the authors contributed to manuscript development and revisions.

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

Declarations

Competing interests The authors declare no competing interests.

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