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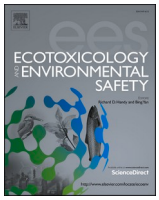

Developing a toolbox of Tier I tests to assess pesticides toxicity on the asymbiotic and symbiotic phases of arbuscular mycorrhizal fungi

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ABSTRACT

Soil microorganisms are a key protection goal in the European Union (EU) pesticide regulatory framework. Arbuscular mycorrhizal fungi (AMF) were identified as good proxies for assessing pesticides toxicity on the soil microbiota. This could involve ecotoxicity testing at the different life stages of AMF. We evaluated the effects of five pesticides (pyraclostrobin, fludioxonil, hymexazol, etridiazole, glyphosate) and a transformation product (AMPA), with distinct mode of action, on the development and functionality of *Rhizophagus irregularis* at the asymbiotic and symbiotic phase using a spore germination assay and a gnotobiotic AMF-host plant system (AMF-sandwich test), respectively. Based on arbuscular colonization in the AMF-sandwich test, fludioxonil was the most toxic (EC_{50} 0.085 mg/L) followed by glyphosate (EC_{50} 2.58 mg/L) and pyraclostrobin (EC_{50} 9.22 mg/L), while etridiazole, hymexazol, and AMPA showed EC_{50} values higher than the highest tested concentration. However, for glyphosate and pyraclostrobin negative effects on symbiosis functioning were observed at lower concentrations than for colonization, as depicted by the expression of plant marker genes and/or P-uptake, suggesting the establishment of non-functional arbuscular symbiosis. The high toxicity of fludioxonil (EC_{50} 0.03 mg/L) and the low toxicity of AMPA (EC_{50} > 432 mg/L) on *R. irregularis* was verified also for the asymbiotic phase via spore germination assay. Comparative tests showed differences in the toxicity of pure active substances and commercial formulations of fludioxonil and pyraclostrobin on the AMF-sandwich test. We propose that the AMF-sandwich system together with the spore germination test could be used as a toolbox for Tier-I assessment of pesticides toxicity on AMF.

1. Introduction

Pesticides, including herbicides, are major agrochemical inputs and constitute important environmental pollutants whose effects often extend beyond the target organisms. Pesticides not only pose risks to human health and the environment but also affect the functionality of soil microorganisms (Ni et al., 2025). The soil microbiota is important for ecosystem homeostasis based on their key functional role on soil fertility (George and Ray, 2023), soil structure (Rillig et al., 2015) and degradation of pollutants (Fenner et al., 2013). Some of these microorganisms (e.g. rhizobia, arbuscular mycorrhizal fungi (AMF)) form symbiotic associations with plants facilitating plant nutrient acquisition and enhancing plant tolerance to biotic and abiotic stress (Trivedi et al., 2020). The European Food Safety Authority (EFSA) identified critical weaknesses and gaps in the assessment of the toxicity of pesticides on soil microorganisms and promoted soil microbes as ecosystem protection goals (Panel et al., 2010). In this frame, EFSA proposed AMF as potent bioindicators to determine the toxicity of pesticides on soil microbiota (Ockleford et al., 2017).

AMF belong to the phylum *Glomeromycota* and are the most widespread plant symbionts, forming symbiotic associations with approximately 72 % of vascular plant species (Brundrett and Tedersoo, 2018). They colonize root cortical cells and improve plant fitness by facilitating mineral nutrient, especially phosphorus (P), and water uptake (Kakouridis et al., 2022) and by enhancing their tolerance to biotic and abiotic stresses (Marro et al., 2022). In return, host plants provide organic carbon to AMF in the form of plant-derived photosynthates and lipids (Lanfranco et al., 2017). Beyond enhancing plant fitness, AMF symbiosis provides vital ecological services such as nutrient transport among plants connected through the AMF hyphal network, soil

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aggregation and stabilization (Rillig et al., 2015), enrichment of soil carbon reservoirs (Fernandez and Kennedy, 2015), facilitation of microbe transportation via their hyphal network (AMF highways) (Vieira et al., 2025), and control of the plant community diversity and productivity (van der Heijden et al., 2008).

The establishment of a functional symbiosis involves distinct biological stages, including the (a) asymbiotic phase which involves spore germination, germ tube elongation and hyphal ramification, (b) the presymbiotic phase which involves AMF-host signaling and hyphopodium formation, and (c) the symbiotic stage which consists of host colonization, arbuscule and vesicle development, nutrient transportation and sporulation (Bonfante and Genre, 2010). During these stages, disturbances such as pesticide exposure can have detrimental effect on symbiosis. The effects of pesticides could be direct (e.g., fungicides) or indirect (e.g., herbicides toxicity on plant hosts). However, the nature of pesticide effects on AMF is often difficult to discern due to their obligatory symbiotic lifestyle and needs combinatory testing approaches that focus on different life stages of the AMF (Sweeney et al., 2022).

Earlier studies have investigated the toxicity of pesticides on AMF at different experimental scales (Buysens et al., 2015; Karpouzas et al., 2014). For example, *in vitro* testing approaches based on the Ri T-DNA-transformed root organ culture (ROC) system allowed the estimation of inhibitory concentration 50 (IC_{50}) values for a range of pesticides (Brundrett and Tedersoo, 2018). Wen et al. (2022) used the bi-compartmental (root and hyphae compartments) ROC system to evaluate the effects of the fungicides fenpropimorph and fenhexamid on the asymbiotic (spore germination, germ tube elongation) and the symbiotic phase (root colonization). Recently, Roshanfekrrad et al. (2025) developed and validated an *in vitro* high-throughput spore germination test to screen pesticides for toxicity on AMF at the asymbiotic germination phase. In another recent study Gkimprixi et al. (2023) used the AMF-sandwich gnotobiotic bioassay, to evaluate the effect of two veterinary medicines on arbuscule colonization and functionality of the AMF *Rhizophagus irregularis* in the legume plant *Lotus japonicus*. This work showed that albendazole did not reduce arbuscular colonization but leads to the formation of arbuscules with impaired functionality. The AMF-sandwich bioassay provides an assessment of the potential effects of pesticides on the symbiotic phase of AMF complementing the spore germination assay (Roshanfekrrad et al., 2025) and the ISO-10832 spore germination test (International Organization for Standardization, 2009), which both assess pesticides effects on the asymbiotic phase of AMF. It is a low cost, easy to implement method that has low instrumentation requirements and hence could be easily adopted by most laboratories.

The main research objective of the current study was to evaluate the potential use of the AMF-sandwich test, along with the spore germination assay, as a toolbox for the early and conservative determination of the toxicity of pesticides on different life stages of AMF (symbiotic and asymbiotic). We consider AMF as valid indicators for the determination of potential adverse effects of pesticides on soil microbiota. To achieve this main goal, we monitored the effects of four fungicides (pyraclostrobin, fludioxonil, hymexazol and etridiazole), one herbicide (glyphosate) and its transformation product AMPA (2-amino-3-(5-methyl-3-oxo-2,3-dihydro-1,2-oxazol-4-yl)propanoic acid) on the symbiotic phase of the AMF model organism *R. irregularis* (MUCL 41833) in the gnotobiotic AMF-sandwich assay. We expanded our testing to the asymbiotic phase and assessed the effects of two of the compounds mentioned above, fludioxonil and AMPA, on *R. irregularis* using a high-throughput spore germination assay. Furthermore, we investigated potential differences in the toxicity between active ingredients (a.i) and commercial formulations of two of the most toxic compounds, pyraclostrobin and fludioxonil.

2. Material and methods

2.1. Pesticides and Arbuscular Mycorrhizal Fungi

Analytical standards of pyraclostrobin (99.9 %, HPC Standards GmbH, Germany), fludioxonil (99.7 %, Sigma-Aldrich, St Gallen Switzerland), hymexazol (87 %, Alfa Aesar, Thermo Fisher Scientific, USA), etridiazole (99.88 %, HPC Standards GmbH, Germany), glyphosate (99.87 %, HPC Standards GmbH, Germany), AMPA (99 %, Alfa Aesar, Thermo Fisher Scientific, USA) were used in both tests. In addition, commercial formulations of pyraclostrobin (Comet®, BASF, Japan) and fludioxonil (Scholar®, Syngenta, Switzerland) were also used in certain tests. The chemical structures and the physicochemical properties of the studied pesticides are given in Table S1.

The AMF species *R. irregularis* MUCL 41833 from the *Glomeromycota in vitro* collection (GINCO) was used in our assays. The fungus was associated with Ri T-DNA transformed hairy roots of *Daucus carota* L. in bi-compartmented Petri plates until a sufficient number of spores was produced (3–6 months) to conduct our assays.

2.2. AMF-sandwich test – experimental setup

L. japonicus L. ecotype Gifu B-129 wild-type was used as the host plant for *R. irregularis* in our sandwich testing system (Gkimprixi et al., 2023). Fig. 1 details the full setup of the testing system. In brief, *L. japonicus* seeds were surface scarified (30 min in H_2SO_4), disinfected (10 min in 20 % bleach) and kept in sterile dH_2O for 12 h at 4 °C. They were then germinated for 10 days at 21 °C in wet sterile filter paper (16 h light, 8 h dark). The plantlets were subsequently embedded in a pair of pre-sterilized nitrocellulose discs arranged in a sandwich configuration (three plantlets per sandwich) and inoculated with 300 spores of AMF that were collected from *in vitro* ROC systems in small gel plugs (free of roots). This sandwich configuration was then placed in magenta boxes (7.62 cm × 7.62 cm × 10.16 cm) containing 360 g of baked sand (180 °C, 18 h) which was irrigated with 60 mL of Long-Ashton (SLA) nutrient solution (0.75 mM $MgSO_4$, 1 mM $NaNO_3$, 1 mM K_2SO_4, 2 mM $CaCl_2$, 3.2 μM Na_2HPO_4, 25 μM FeNa-EDTA, 5 μM $MnSO_4$, 0.25 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 25 μM H_3BO_3, 0.1 μM Na_2MoO_4 in dH_2O), either amended or not amended with pesticides. Non-polar compounds (pyraclostrobin, fludioxonil, hymexazol, etridiazole) were first dissolved in dimethyl sulfoxide (DMSO) (10.000 mg L^{-1}) and then added in the SLA medium to achieve the desired pesticide concentrations, with the concentration of DMSO in the SLA medium never exceeding 0.1 %. In contrast, polar compounds (glyphosate, AMPA) and pesticide commercial formulations were dissolved directly to the SLA medium. The pesticides studied were chosen based (a) primarily on previous findings on spore germination assays described in Roshanfekrrad et al. (2025) aiming to include compounds with distinct mode of action that exhibited a range of toxicity on the spore germination assay and (b) secondly on the regular use of these compounds in the EU. The tested concentrations per pesticide (Table S2) and the range was selected based on the maximum recommended agronomic dose of the pesticides studied. The full range of pesticide concentrations was tested for the determination of effects on AMF colonization levels and shoot P concentration, while selected concentrations from those above were tested for monitoring effects on the expression of plant marker genes associated with mycorrhizal symbiosis. The system was incubated at 24–25 °C (16 h light, 8 h dark) and harvested at four or five weeks depending on the measurement employed.

The concentrations of the studied pesticides, except of glyphosate and AMPA for which no analytical method was available, in the SLA medium applied to the sandwich system were determined via high-performance liquid chromatography (HPLC). Briefly, pesticide residues were extracted by mixing 1:1 by volume aliquots of the SLA medium with acetonitrile (pyraclostrobin, etridiazole) or methanol (fludioxonil, hymexazol), vortexing for 1 min and then directly analyzed

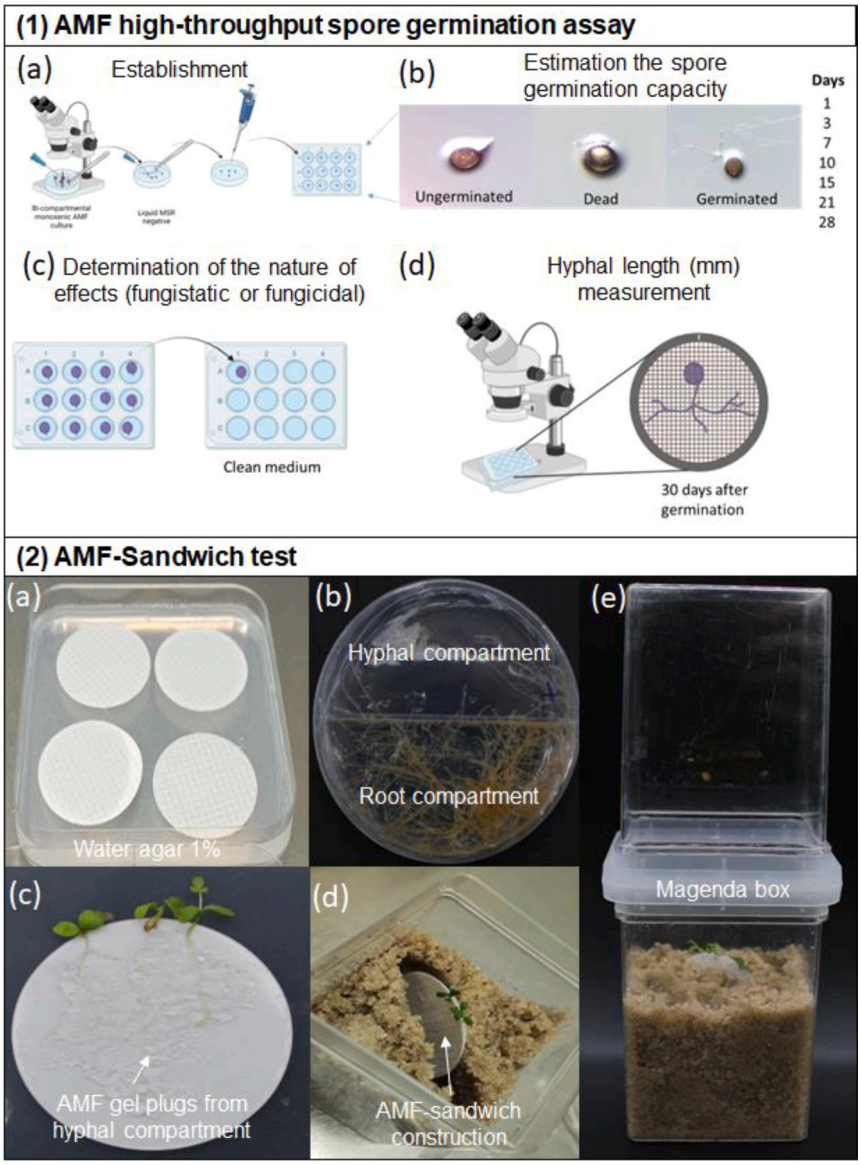

Fig. 1. A schematic representation of the (1) AMF high-throughput spore germination assay and the (2) AMF-sandwich test. In (1) (a) Spores of *Rhizophagus irregularis*, obtained from an in vitro bi-compartmental culture, were transferred in 12 well-microplates. Two mL of MSR medium was fortified with pesticides at five concentration levels and before solidification with Gerlite were added in each well. Viable spores were transferred individually with a micropipette to each well and were incubated at 27°C in the dark; (b) Spore germination was assessed at 1, 3, 7, 10, 15, 21 and 28-days post-inoculation under binocular microscope; (c) Un-germinated spores were cleaned with pesticide-free MSR and transferred onto solidified MSR medium without pesticides to evaluate their re-germination capacity and determine the nature of the pesticide effects (fungistatic or fungicidal); (d) The hyphae length of germinated spores is measured at 30 days post germination. In (2) (a) Nitrocellulose filter papers are pre-wet into autoclaved water-agar, (b) *R. irregularis* spores are isolated from in vitro bi-compartmental culture (root compartment (root and AMF), hyphal compartment (AMF, free of root)) systems of transformed roots of *Daucus carota*, small gel plugs from the hyphal compartment were cut and quantified under binocular stereo microscope, (c) three 10-day old plantlets of *Lotus japonicus* are placed on a pre-wet filter and are inoculated spores of *R. irregularis* and the filters are covered by a second filter forming a sandwich configuration, (d) baked sand were placed into magenta boxes and were irrigated with Long-Ashton nutrient solution (SLA), either amended or not amended with pesticides. The sandwich construct is placed in the magenta box filled with the SLA-treated baked sand, (e) plants are grown in double magenta boxes for 5 weeks at 24–25 oC (16 h light, 8 h dark).

in a Shimadzu HPLC-DAD system equipped with a Grace 297 Smart RP C18 (150 mm×4.6 mm) as described in Table S3.

2.3. *Effects of pesticides on AMF root colonization and phosphorus concentrations*

The percentage of arbuscular colonization on *L. japonicus* roots and the P concentrations in the aboveground plant parts were measured in five-week treated plants. It should be noted that concentrations that cause phytotoxicity, exhibited as stunted growth, dried or yellowish plantlets, and significantly lower plant biomass measurements compared to the control at harvest, were excluded from the analysis. AMF colonized roots were treated with KOH 10 % for 40 min before staining with 5 % ink in a 5 % acetic acid solution (Vierheilig et al., 1998), and colonization levels were determined by optical microscope. The roots of three plantlets (approximately 20 cm in length) were used for each slide preparation. For colonization quantification, the percent (%) of arbuscular colonization in at least 100 eyepieces per slide was determined. Five slides were examined per treatment, corresponding to five biological replicates.

Above ground shoots from five-week treated *L. japonicus* plantlets were dried at 70 °C for 48 h to measure the dry biomass. Ten to twenty

milligrams of the dried shoot material were ashed to 550 °C. The resulting ash was solubilized with 1 mL of aqua regia, a 3:1 mixture of HCl and HNO_3. The extract was then diluted to 4 mL with distilled water. Total P concentration in shoots was determined spectrophotometrically at 880 nm using the Murphy and Riley (Murphy and Riley, 1962) color reaction method.

2.4. Effect of pesticides on the functionality of the AMF established symbiosis

Based on the results of plant biomass and colonization measurements, a second experiment was performed, following the same setup, to assess the effect of the six pesticide compounds on the functionality of the AMF-plant symbiosis. AMF-sandwich systems were treated with the six different pesticides at concentrations that showed low or no effect on AMF colonization (Table S2). Plants were harvested four weeks after establishment, and the expression levels of three AMF symbiosis-induced plant genes in roots were quantified using RT-qPCR. The transcriptional levels of *LjSbtM1* (XM_057587751.1) serve as indicators of normal stage transition during arbuscules development, while the transcriptional levels of *LjPT4* (XM_057584022.1) and *LjAMT2;2* (XM_057567303.1) are used as indicators of the active plant uptake of inorganic P and nitrogen (N), respectively, from the peri-arbuscular space (Gkimprixi et al., 2023).

Plant root RNA was extracted with a modified Lithium Chloride-TRIzol LS (Invitrogen) method (Holt et al., 2015) and quantified with nanodrop (Quawell). RNA was then treated with DNAse I (Thermo Scientific) according to the manufacturer instructions. The effectiveness of DNA removal was checked via PCR using Ubiquitin (UBQ) (XM_057568040.1) primers (Table S3) with *L. japonicus* DNA serving as a positive control (Delis et al., 2011). RNA samples were then subjected to One-Step qPCR using 10 ng of RNA (1 μl) with the Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs). The total volume of the reaction was 10 μl. The thermocycling parameters consisted of incubation at 55°C for 10 min for reverse transcription (cDNA synthesis), 95°C for 1 min, followed by 39 cycles of 95°C for 10 s and 58–60 °C for 30 s (amplification). The expression levels of the target genes were normalized with the use of two reference genes: *L. japonicus ATP SYNTHASE2* (*LjATP2*) (M_057567303.1) and *L. japonicus PROTEIN PHOSPHATASE2a* (*LjPP2a*) (XM_057591089.1). RT-qPCR reactions were performed in a BioRad CFX Connect light cycler (BioRad). The sequences of the primers used are given in Table S4.

2.5. AMF spore germination test – experimental setup

We evaluated the effect of five concentrations (Table S2) of fludioxonil and AMPA on spore germination and germ tube elongation of the AMF strain *R. irregularis* MUCL 41833 and further examined the nature of the effect (fungistatic or fungicidal), as described by Roshanfekrrad et al. (2025). Briefly, viable spores of *R. irregularis* MUCL 41833 were transferred individually with a micropipette into separate wells in 12-wells microplates (36 spores considered) and incubated at 27°C in the dark (Fig. 1). Two mL of sterile modified Strullu and Romand medium (MSR) (Declerck et al., 2005) lacking sucrose and vitamins, were amended with five concentrations of the two pesticides that were poured before solidification. Fludioxonil was dissolved in acetone before its addition to the MSR medium, while AMPA was added as water solution to the unsolidified MSR medium. Control treatments included MSR medium amended with either acetone (0.5 mL/L) or ddH_2O without pesticides. Spore germination was assessed at 1, 3, 7, 10, 15, 21 and 28-days post-inoculation under binocular microscope at 45X magnification. A minimum germination rate of 75 % in the control after 28 days of growth was a threshold used to consider the assay as valid, as proposed by Mallmann et al. (2018). It is worth noting that our dH_2O and acetone controls reached 100 % and 97 % germination rates at 28 days. The EC_{50} values (i.e. concentration of the pesticide that reduces half of the germination) were calculated based on data measured at 28 days (Roshanfekrrad et al., 2025). The hyphae length of germinated spores was measured 30 days post germination using the method described by Declerck et al. (2003), where a fine grid (1 mm^2 squared) is placed under the microplate containing the germinated spores, and the hyphae is observed using a binocular microscope. Hyphal length was calculated using the following formula: $(L = ((N \times \pi \times A))/(2 \times H))$ where L is the hyphal length (mm), N is the number of intersections, A is the area (mm^2), and H is the length of the grid (mm).

To determine the fungicidal or fungistatic effect (Buysens et al., 2015; Zocco et al., 2008) of the tested pesticide, treatments where spore germination was inhibited by more than 50 % after 28 days were considered. Non-germinated spores were transferred to new microplates containing pesticide-free MSR gel medium lacking sucrose and vitamins. Spore germination was monitored over a period of one month, and pesticide concentrations were classified into five categories as completely fungicidal if 0 % of spores germinated, as highly fungicidal if spore germination ranged from 1 % to 40 %, as intermediately toxic if the % of spores germination ranged between 41 % and 60 %, as strongly fungistatic or totally fungistatic if the % of spores germination was 61–99 % or 100 % respectively.

2.6. Calculation of the effective concentrations 50 % (EC_{50}) for each pesticide

The pesticide concentration achieving 50 % reduction (EC_{50}) of root AMF colonization was determined according to Papadopoulou et al. (2020) using the dose-response curve (drc) v3.0–1 package (Ritz and Streibig, 2005) of the R software. The determination of the EC_{50} from the spore germination assay was performed using the three-parameters log-logistic model which was the best compromise among tested models for comparing endpoint values as described in Roshanfekrrad et al. (2025). Dose response modelling was performed using normalized data from spore germination at 28 days, as derived from the spore emergence test, and arbuscule percent colonization, as derived from the AMF-sandwich test. The concentrations were divided by the mean value of the corresponding control.

2.7. Data analysis

Data were checked for normality and homoscedasticity using Shapiro and Levene's tests, respectively. The data from the sandwich test were analyzed using ANOVA and Tukey's honestly significant difference (HSD) post hoc test. In cases where the data from the sandwich test did not follow normal distribution, the Kruskal-Wallis test was used ($p \leq 0.05$). All quantitative data are presented as mean ± standard error (SE). Statistical analysis and graphs were made in GraphPad prism 8.0.2 (GraphPad Software San Diego, California USA).

3. Results

3.1. Pesticides effects on plant biomass

Hymexazol did not cause any significant biomass reduction at the range of concentrations tested (Fig. 2a). Etridiazole showed no phytotoxicity at concentrations ≤ 5.76 mg/L (Fig. 2b). Regarding glyphosate, we noted strong phytotoxicity at 21.6 mg/L but no phytotoxicity and biomass reduction at concentrations ≤ 4.32 mg/L (Fig. 2c). Similarly, AMPA was phytotoxic at concentration ≥ 43.2 mg/L (Fig. 2d). Pyraclostrobin, applied either as pure a.i. (Fig. 2e) or as its commercial formulation Comet® (Fig. 2g), showed no significant effects on plant biomass. Fludioxonil, when applied as pure a.i. showed significant biomass reduction ($p < 0.05$) at the higher concentration level tested (39 mg/L) (Fig. 2f), unlike its commercial formulation Scholar®, which had no negative effect on plant biomass (Fig. 2h).

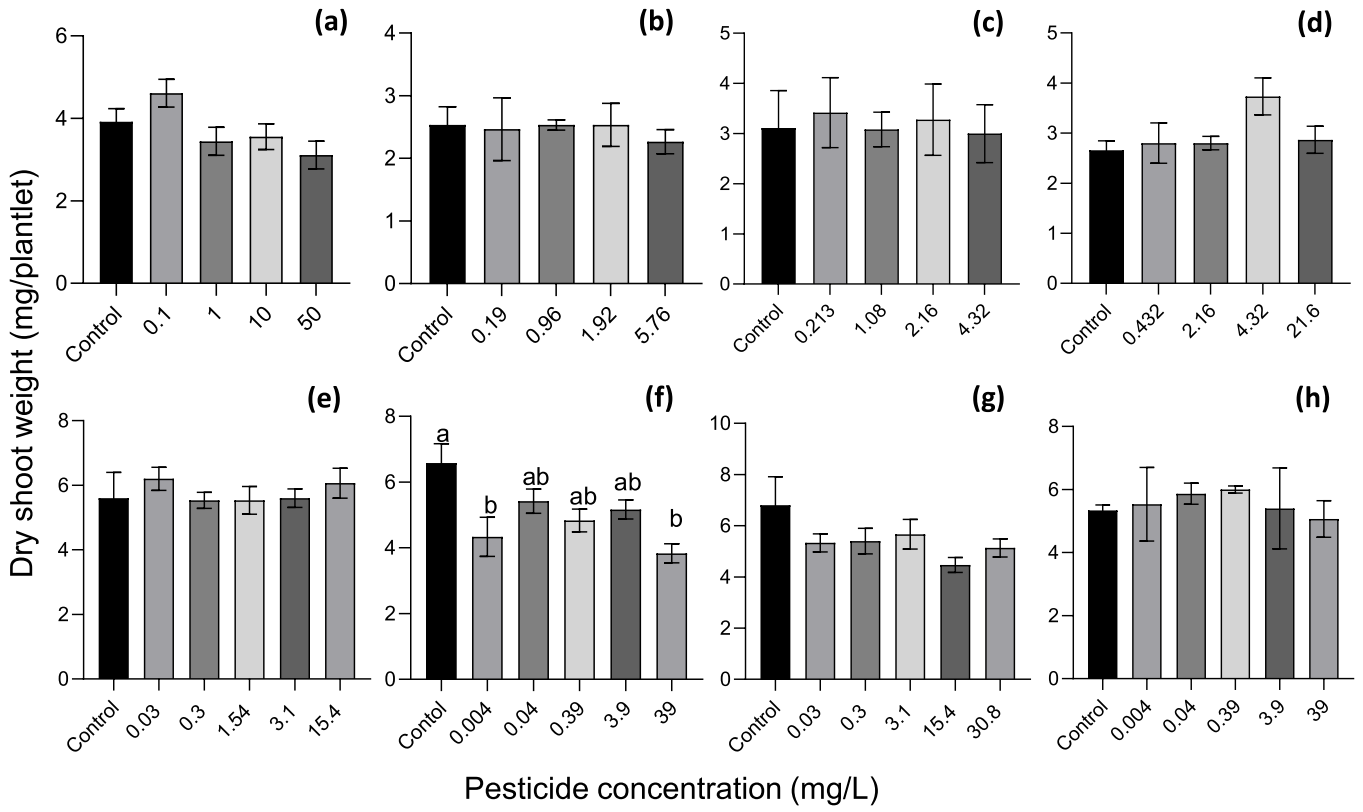

Fig. 2. *Lotus japonicus* aboveground shoot dry biomass (mg/plantlet, mean ± Standard error (SE)) in the AMF-sandwich test. Plants were treated with different concentrations of the pesticides (a) hymexazol (b) etridiazole, (c) glyphosate, (d) AMPA, (e) pyraclostrobin (f) fludioxonil (g) Comet® (pyraclostrobin formulation), (h) Scholar® (fludioxonil formulation) or remained untreated (control). DMSO or ddH_2O were used instead of pesticides in the control treatment (ddH_2O for AMPA and glyphosate and DMSO for the other pesticides). Bars with the same letters are not significantly different (Tukey's test, $p \leq 0.05$ n = 4–5 AMF-sandwich).

3.2. Pesticides effects on arbuscular colonization levels

Hymexazol and AMPA did not affect arbuscular colonization at the concentrations tested (Figs. 3a and 3d). Etridiazole had a significant negative effect ($p \leq 0.05$) on arbuscular colonization at concentrations > 1.92 mg/L (Fig. 3b). Glyphosate reduced arbuscular colonization levels in a dose-dependent manner, with significant reductions observed only at the highest concentration level of 4.32 mg/L ($p < 0.05$) (Fig. 3c). Pyraclostrobin imposed negative effects on arbuscular colonization, which followed a dose-dependent pattern, regardless of the form used (a.i. or formulation), with significant reduction in colonization ($p < 0.05$) observed at levels ≥ 3.1 mg/L (Figs. 3e and 3g). Fludioxonil also showed a clear dose-dependent adverse effect with significant reduction ($p < 0.001$) in arbuscular colonization observed at concentrations ≥ 0.39 mg/L (Figs. 3f and 3h).

Based on the data of arbuscular colonization, EC_{50} values per pesticide were calculated (Table 1). For hymexazol, AMPA and etridiazole, EC_{50} values exceeded the highest tested concentration (that showed no biomass reduction), which were 50, 21.6 and 5.96 mg/L respectively. Amongst the other tested pesticides, fludioxonil as a.i. showed the lowest EC_{50} value (0.085 mg/L) followed by glyphosate (2.58 mg/l) and pyraclostrobin (9.22 mg/L). When compared with the EC_{50} values obtained by testing the commercial formulations of the two fungicides, we noted a higher EC_{50} value for fludioxonil (0.58 mg/L) and a lower EC_{50} value for pyraclostrobin (2.54 mg/L).

3.3. Pesticides effects on aboveground shoot phosphorus concentrations

Hymexazol (Fig. 4a), etridiazole (Fig. 4b) and glyphosate (Fig. 4c) did not show any significant effect on P concentrations in aboveground plant tissues. In contrast, AMPA showed a significant reduction in the P concentration at 4.32 mg/L, although this effect did not follow a dose-dependent pattern (Fig. 4d). When used as a.i., pyraclostrobin imposed significant reductions in the P concentration of plants at concentration levels of 1.54 and 15.4 mg/L ($p < 0.05$) but not at 3.1 mg/L, denoting deviation from a dose-depended pattern (Fig. 4e). Conversely, when used as Comet® (Fig. 4g), no significant effects on P concentration were observed. Finally, fludioxonil, regardless of the form used, showed a significant negative effect ($p < 0.05$) on the shoot P levels at concentrations ≥ 0.39 mg/L (Figs. 4f and 4h).

3.4. Pesticides effects on arbuscules development and functionality

Hymexazol and AMPA showed no significant negative effects on the expression of the marker genes (Figs. 5a and 5d). Instead, a significant stimulation of the expression of *LjPT4* and *LjSbtM1* was observed in the plants treated with 10 mg/L hymexazol. In contrast, etridiazole significantly reduced ($p < 0.05$) the expression levels of *LjAMT2;2* and *LjSbtM1* at concentrations ≥ 0.96 mg/L and of *LjPT4* at the highest tested concentration of 5.76 mg/L (Fig. 5b). Glyphosate had a dose-dependent negative effect on the expression levels of marker genes (Fig. 5c). It significantly reduced the expression of *LjAMT2;2* and *LjPT4* at concentrations ≥ 2.16 mg/L ($p < 0.05$) and the expression of *LjSbtM1* at the highest tested concentration of 4.32 mg/L ($p < 0.05$).

When comparing the effects of pyraclostrobin and fludioxonil, as a.i. or commercial formulations, on the expression of the marker genes, differences were observed. Pyraclostrobin imposed a dose-dependent reduction in the expression levels of all three genes. When applied as a.i., pyraclostrobin significantly decreased the expression levels of *LjAMT2;2* *($p < 0.05$)* and *LjPT4* ($p < 0.001$) at concentrations ≥ 1.54 mg/L, and of *LjSbtM1* at concentrations ≥ 3.1 mg/L ($p < 0.05$) (Fig. 5e). As Comet®, pyraclostrobin significantly reduced ($p < 0.05$)

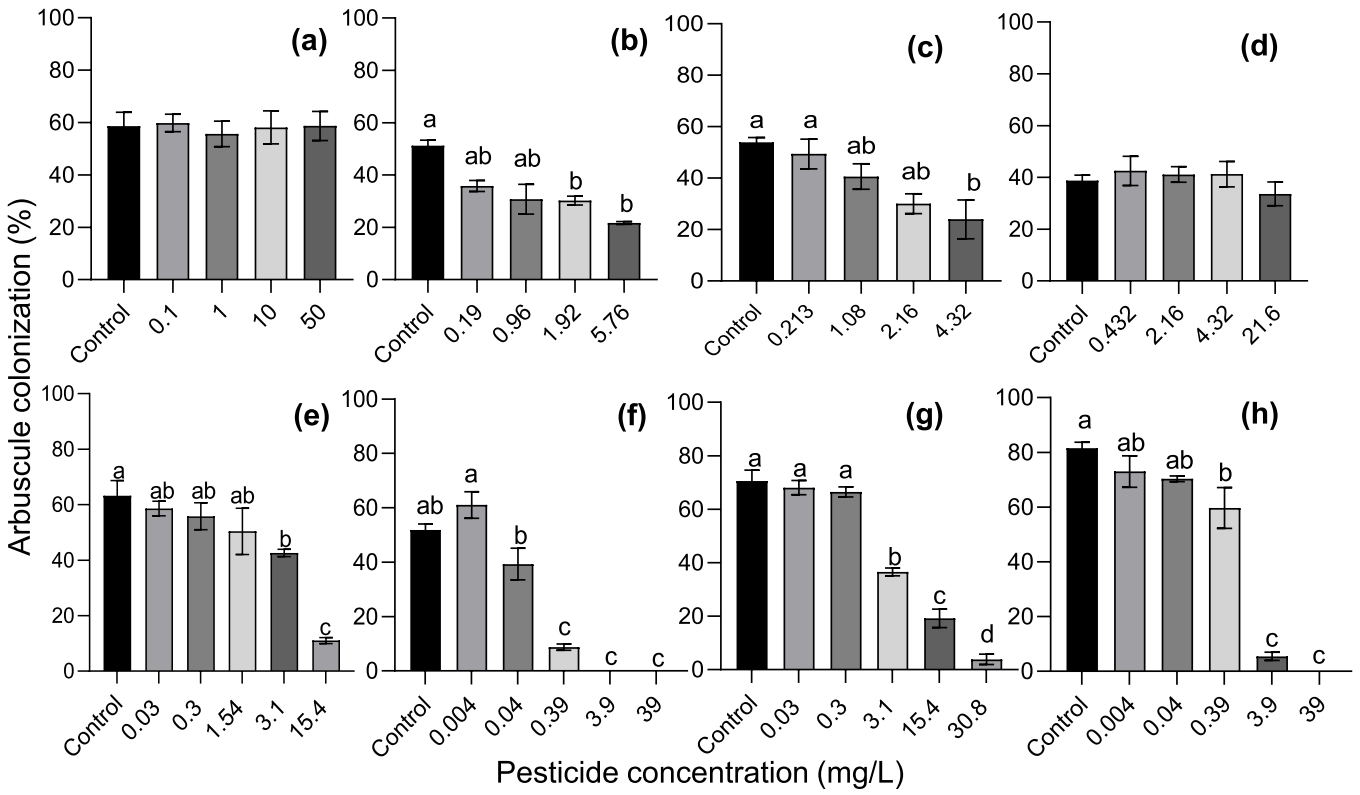

Fig. 3. Arbuscular colonization levels (%, mean + Standard error (SE)) in the roots of *L. japonicus* plants in the AMF-sandwich test. Plants were treated with different concentrations of the pesticides (a) hymexazol (b) etridiazole, (c) glyphosate, (d) AMPA, (e) pyraclostrobin (f) fludioxonil (g) Comet® (pyraclostrobin formulation), (h) Scholar® (fludioxonil formulation) or remained untreated. DMSO or ddH2O were applied instead of pesticides in the control treatment (ddH2O for AMPA and glyphosate and DMSO for the other pesticides). Bars with the same letters are not significantly different (Tukey's test, $p < 0.05$).

Table 1
Mean EC_{50} values (mg/L) of the tested pesticides, calculated based on arbuscular colonization levels derived from the AMF-sandwich test and the spore germination test. Double asterisks denote that EC_{50} values were higher than the highest pesticide concentration tested. The levels of the different tested pesticides expected to be found in soil solution upon application of the maximum agronomic recommended dose are also presented. They were calculated as described in Roshanfekrrad et al. (2025).

Compounds	Recommended field concentration (mg/L)	EC_{50} (mg/L) AMF-sandwich	EC_{50} (mg/L) Spore germination
Hymexazol	0.56	> 50**	
Etridiazole	9.6	> 5.96**	
Glyphosate	21.6	2.58	
AMPA	21.6	> 21.6**	> 432**
Fludioxonil	3.85	0.085	0.03
Pyraclostrobin	3.08	9.22	
Fludioxonil Scholar®	3.85	0.58	
Pyraclostrobin Comet®	3.08	2.54	

the expression of the *LjPT4* gene at concentrations ≥ 3.1 mg/L, while the expression of the other two marker genes was significantly impaired only at the highest tested concentration of 15.4 mg/L (Fig. 5g). Regarding fludioxonil, when applied as a.i., the expression of all three marker genes was significantly reduced ($p < 0.0001$) only at the highest dose tested (0.39 mg/L). Conversely, its application as Scholar® led to a significant suppression of the expression ($p < 0.05$) of *LjAMT2;2* at concentrations ≥ 0.039 mg/L (Fig. 5h), while the expression of the other two marker genes was significantly impaired ($p < 0.01$) only at concentrations ≥ 0.39 mg/L. Besides the negative effects of Scholar® on gene expression, we noted an advent in the expression of all three marker genes at 0.004 mg/L, the lowest concentration tested. However, this positive effect was significant ($p < 0.05$), relative to the control, only for the *LhSbtM1*.

3.5. Pesticides effects on spore germination and germ tube elongation

Fludioxonil showed a clear dose-dependent effect on spore germination with a significant reduction at concentrations ≥ 0.04 mg/L ($p < 0.001$) (Fig. 6a). Interestingly, fludioxonil at the lowest dose (0.004 mg/L) significantly increased germ tube elongation, indicating a hormetic response pattern, whereas strong inhibition of germ tube elongation was observed at higher concentrations (Fig. 6c). AMPA did not show a dose-response effect on spore germination (Fig. 6b) and germ tube elongation (Fig. 6d). Based on these data, the EC_{50} values derived for fludioxonil and AMPA were 0.03 mg/L and > 432 mg/l respectively (Table 1).

We further determined the nature of the toxicity effects only for fludioxonil, since we did not observe any ungerminated spores for AMPA. The effect of fludioxonil on spore germination varied at the different concentrations tested. It was classified as completely or highly fungicidal at levels of 7.7 and 3.9 mg/L respectively and as strongly fungistatic at the lower concentrations tested (0.39 and 0.039 mg/L) (Table S5).

4. Discussion

Several studies have assessed the toxicity of pesticides on AMF, using *in vitro* and *in soil* experimental approaches. Under *in vitro* cultivation conditions, these studies focused on pesticide effects on various stages of the AMF life cycle, including spore germination (Roshanfekrrad et al., 2025; Zocco et al., 2008) anastomosis (Rodriguez-Morelos et al., 2023a),

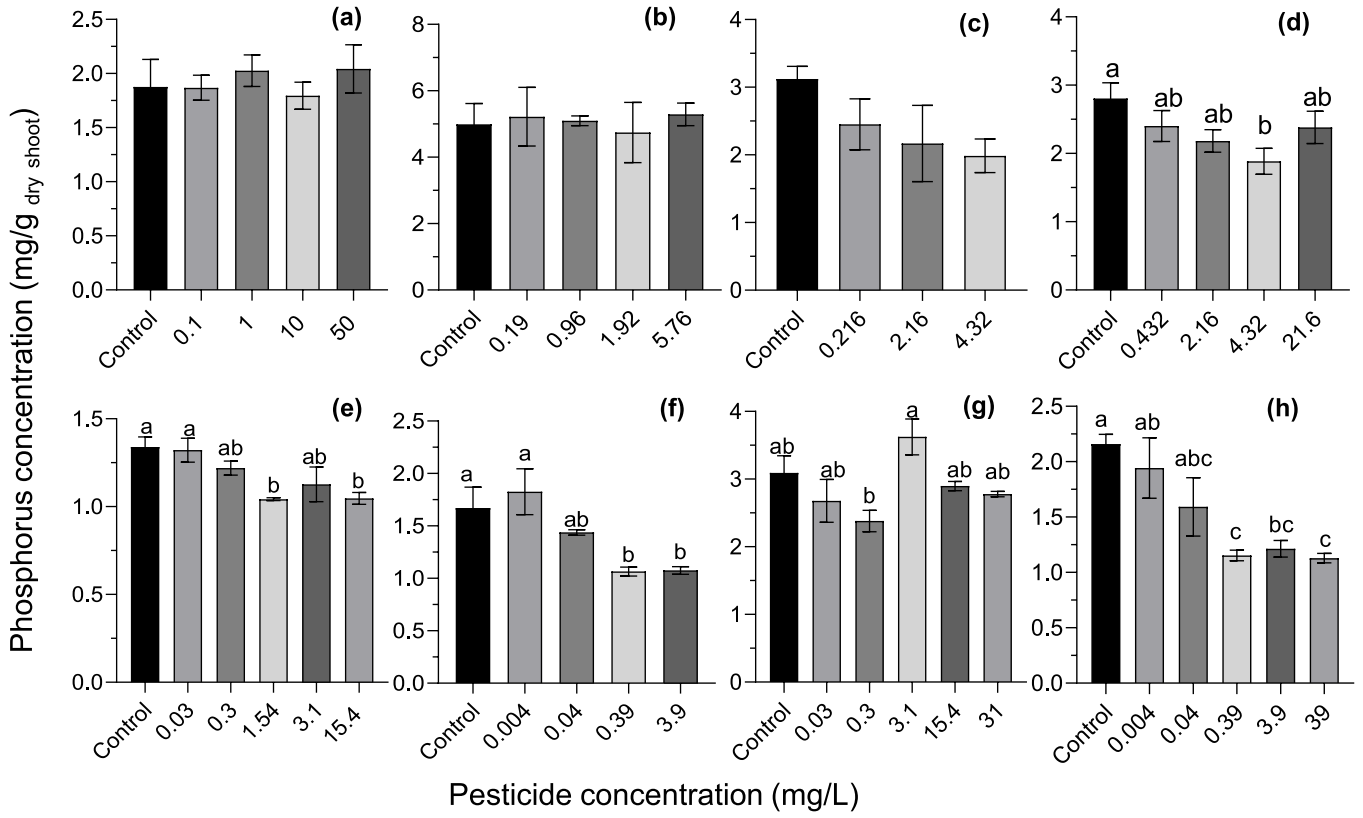

Fig. 4. Phosphorus concentrations (mg/g dry shoot, mean + Standard error (SE)) in the aboveground shoots of *L. japonicus* plants in the AMF-sandwich test. Plants were treated with different concentrations of the pesticides (a) hymexazol (b) etridiazole, (c) glyphosate, (d) AMPA, (e) pyraclostrobin (f) fludioxonil (g) Comet® (pyraclostrobin formulation), (h) Scholar® (fludioxonil formulation) or remained untreated (control). DMSO or ddH2O were used as control treatment (ddH2O for AMPA and glyphosate and DMSO for the other pesticides). Bars with the same letters are not significantly different (Tukey's test, $p < 0.05$ n = 4 AMF-sandwich).

hyphal healing mechanism (Rodriguez-Morelos et al., 2021), colonization capacity, phosphorus transportation (Zocco et al., 2011) and sporulation (Bastogne et al., 2024). In addition, the impact of pesticides on natural soil AMF assemblages has been evaluated in greenhouse (Karpouzas et al., 2014) and field experiments (Hardy et al., 2025). However, the toxicity of pesticides derived by *in vitro* and *in soil* tests remains largely disconnected. Gkimprixi et al. (2023) developed a gnotobiotic experimental setup, to assess the impact of veterinary medicines albendazole and invermectin on *R. irregularis*. This test system allowed to determine a range of toxicity endpoints, structural like arbuscular colonization, and functional like phosphorus uptake and expression of symbiosis-induced plant genes (*LjPT4, LjAMT2;2, LjSbtM1)*. However, it did not consider the effects of toxicants on the asymbiotic spore germination stage. Here, we evaluated the potential of the AMF sandwich system to be used as part of a toolbox of conservative, Tier I testing systems that could provide early warnings about the potential toxicity of pesticides on AMF. This will prevent unnecessary testing at increasing complexity soil-based Tier II testing systems in the frame of a tiered risk assessment approach, as outlined by Karpouzas et al. (2022).

4.1. Toxicity patterns of pesticides on AMF at the symbiotic and asymbiotic phase

Six pesticides with varying modes of action, commonly used in European agriculture, were tested: hymexazol, etridiazole, glyphosate, AMPA, pyraclostrobin and fludioxonil. Glyphosate is a non-selective systemic herbicide and glycine derivative that inhibits the 5-enolpyruvylshikimate-3-phosphate synthase. Its impact on AMF has been extensively studied, with most studies indicating direct and/or indirect negative effects on AMF spore germination (Roshanfekrrad et al., 2025) and sporulation (Bastogne et al., 2024). In a recent study that focused on the asymbiotic phase, Roshanfekrrad et al. (2025), demonstrated that glyphosate inhibited spore germination of *R. irregularis (*MUCL 41833, EC_{50} 40.6 ± 3.33 mg/L*), Rhizophagus intraradices (*MUCL 49410 EC_{50} 259.2 ± 11.2 mg/L*) and Rhizophagus aggregatus (*MUCL 49408 EC_{50} 75.6 ± 6.31 mg/L*),* but not of *Rhizophagus clarus* (MUCL 46238) (EC_{50} >432 mg/L). On the other hand, the determination of glyphosate impact on AMF colonization during the symbiotic phase is a challenge due to its high phytotoxicity. Previous studies have suggested that the reduced AMF colonization was attributable to glyphosate phytotoxicity rather than on direct effects on AMF (Carvalho et al., 2014), although a more recent study by Bastogne et al. (2024) did not exclude possible direct effects of glyphosate on *Rhizophagus* species in plant roots. In our AMF-sandwich assay, a dose range that did not exhibit phytotoxicity was considered, hence indirect effects of glyphosate were excluded. Glyphosate reduced root colonization by *R. irregularis* at a concentration of 4.32 mg/L, the highest tested concentration, while it impaired the expression of genes involved at N (*LjAMT2;2)* and P uptake (*LjPT4)* at a lower concentration (2.16 mg/L). These results suggest that although *R. irregularis* is capable of colonizing the roots of *L. japonicus* at glyphosate concentrations of 2.16 mg/L, the functionality of the symbiosis is impaired. It also underlines the potential of molecular analysis to reveal inhibitory effects that will be otherwise overlooked based on colonization levels. The EC_{50} value of glyphosate, as derived by the AMF sandwich test (2.58 mg/L), is a fold lower than the concentrations of glyphosate expected to be found in the soil solution upon application of the recommended dose rate (21.6 mg/L) and within the range of concentrations of glyphosate found in agricultural soils in monitoring studies in Europe (0.25–10.25 mg/L soil solution as calculated from soil concentrations of 0.05–2.05 mg/kg measured) (Silva et al., 2018) and elsewhere (20 mg/L in soil solution corresponding to measured soil

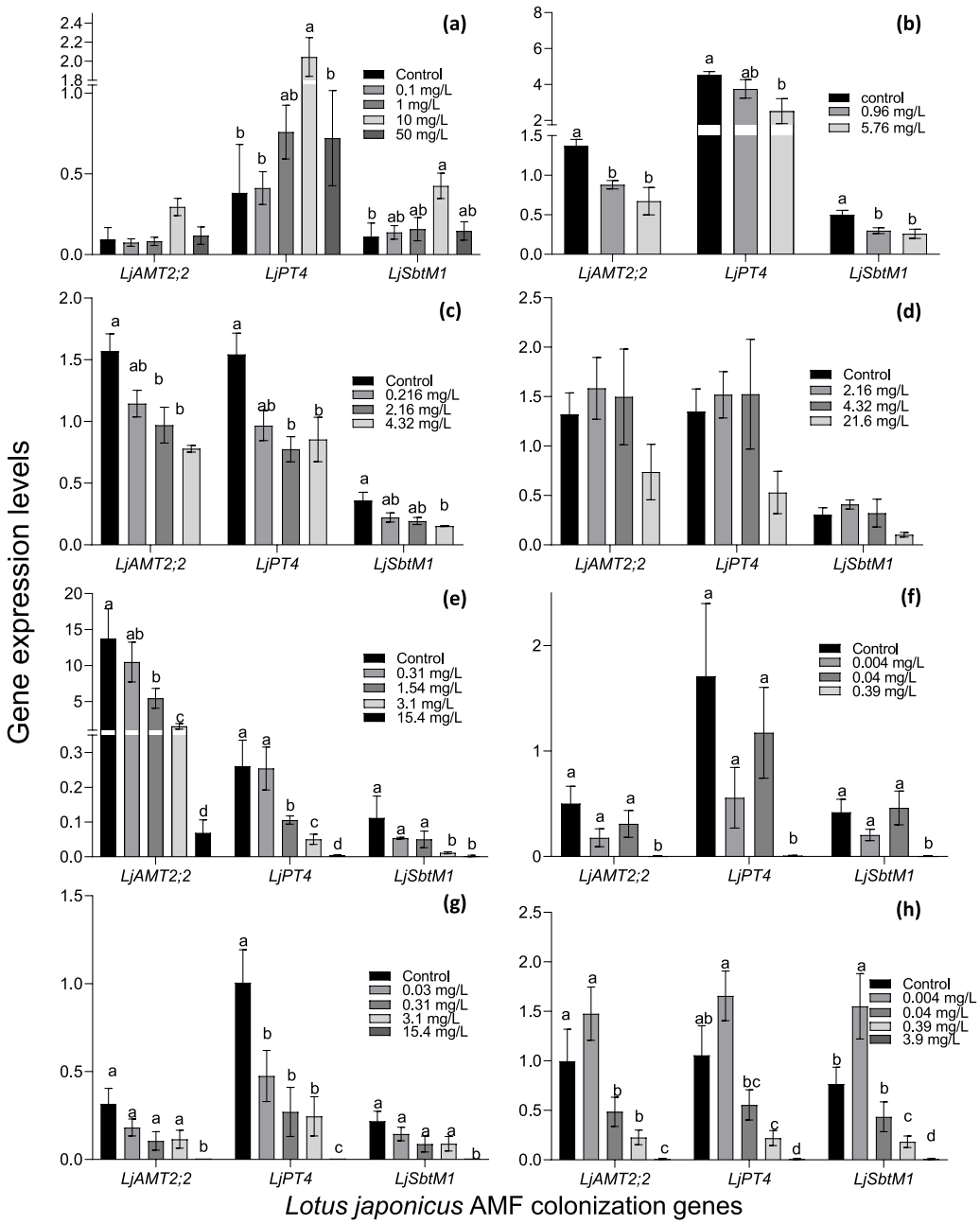

Fig. 5. The effects of different concentrations of pesticides (a) hymexazol (b) etridiazole, (c) glyphosate, (d) AMPA, (e) pyraclostrobin a.i, (f) fludioxonil a.i, (g) Comet® (pyraclostrobin), (h) Scholar®(fludioxonil) on the expression levels (mean + Standard error (SE)) of plant marker genes *LjAMT2;2* (Ammonium transporter), *LjPT4* (phosphorus transporter) and *LjSbtM1* (arbuscule development transcription factor) in the AMF-sandwich test. Expression levels were also determined in untreated plants where ddH2O (AMPA and glyphosate) or DMSO (all other pesticides) were used instead of pesticides. Bars with the same letters are not significantly different (graphs a, b, c, d, f, h were analyzed using Tukey's test, $p < 0.05$, for graphs e, g Kruskal-Wallis, $p < 0.05$ n = 3–4 AMF-sandwich).

concentrations of 4 mg/kg) (Primost et al., 2017), highlighting the potential risk for the soil AMF community by the application of this herbicide (Bastogne et al., 2024). On the other hand, the major transformation product of glyphosate, AMPA, did not affect neither the colonization capacity of *R. irregularis*, nor the symbiosis functioning in the AMF-sandwich test and did not show any toxicity on spore germination at the studied concentrations. Previous studies by Wan et al. (1998) showed that AMPA inhibited *Glomus intraradices* (now re-classified as *R. intraradices* or *R. irregularis)*, extraradical mycelium growth (IC_{50} 5.1 mg/L) and sporulation (IC_{50} 5.5 mg/L). This inhibition effect may be host-driven (*Daucus carota)*, as *R. intraradices* was not affected by AMPA in the spore germination test (Roshanfekrrad et al., 2025) at concentrations higher than those tested by Wan et al. (1998). Still, it cannot be excluded that the differences between our study and the study of Wan et al. (1998) could be just the result of the measurement of distinct endpoints that differ substantially in their metabolism and functionality (i.e. extra-radical mycelium and spore germination) (Maclean et al., 2017).

Etridiazole is a thiadiazole fungicide that inhibits ergosterol biosynthesis in fungi. Etridiazole showed limited effects on the spore germination capacity of *R. irregularis* (Roshanfekrrad et al., 2025). In our study though, etridiazole significantly reduced arbuscular colonization and the functionality of the symbiosis, as depicted by the reduced expression of plant genes associated with AMF symbiosis. Nevertheless, this negative effect was not reflected in P concentration in plants, in line with the expression of the *LjPT4* gene, associated with P uptake by

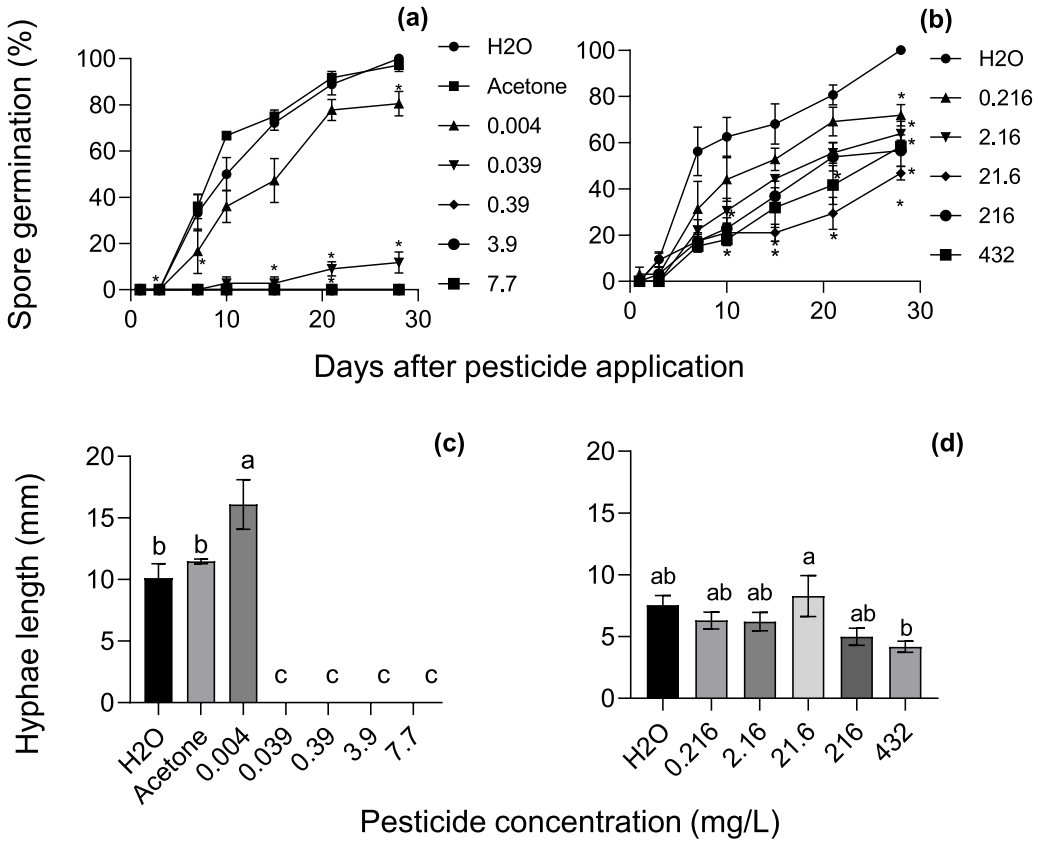

Fig. 6. The effects of fludioxonil and AMPA on spore germination (a and b) and germ tube elongation (c and d) of the arbuscular mycorrhizal fungus *R. irregularis* (mean + Standard error (SE)). Asterisk in the spore germination plots (a and b) show significant differences with the control according to the Pearson's Chi-squared test ($p \leq 0.05$; n = 36 spores). In the bar charts for germ tube elongation (c and d), bars designated by the same letters are not significantly different (Tukey's test, $p < 0.05$ n = 36spores).

arbuscules, which was the least affected marker gene. Collectively our findings, along with those of Roshanfekrrad et al. (2025), suggest that etridiazole exhibits higher toxicity to *R. irregularis* at the symbiotic rather than at the asymbiotic phase. Previous studies with other pesticides of the sterol-biosynthesis inhibition group (i.e. propiconazole, fenpropimorph) reported strong alterations in the sterol biosynthesis of both partners in the AM fungal symbiosis that might exacerbate the effects of fungicides like etridiazole at the symbiotic compared to the asymbiotic phase of the fungus (Calonne et al., 2012). On the other hand, the systemic fungicide hymexazol, an isoxazole with a different mode of action (inhibition of nucleic acid synthesis), had no effect on the asymbiotic (Roshanfekrrad et al., 2025) as well as the symbiotic phase of *R. irregularis*.

Pyraclostrobin is a multipurpose strobilurin fungicide that acts as inhibitor of the Complex III in the fungal respiratory chain (Dominguez et al., 2021). It has shown high inhibitory activity on *Rhizophagus* species and particularly *R. irregularis* (MUCL 41833, EC_{50} 0.25 ± 0.03 mg/L) in the spore germination test (Roshanfekrrad et al., 2025). In the AMF-sandwich test, pyraclostrobin negatively affected arbuscule colonization at concentrations ≥ 3.1 mg/L (EC_{50} 9.22 mg/L). However, it significantly impaired the functionality of AMF at lower concentration (≥ 1.54 mg/L) as denoted by the reduced P concentration in plant tissues and the expression of symbiosis-relevant genes, in line with earlier studies by Rodriguez-Morelos et al. (2023b) with axoxystrobin, another strobilurin fungicide. These results indicate that pyraclostrobin affects *R. irregularis* both at the asymbiotic and the symbiotic phase by impairing nutrient transportation and functioning of AMF arbuscule organelles.

Fludioxonil, a fungicide from the phenylpyrrole class, inhibits the phosphorylation of glucose (transport association proteins) (Wen et al., 2022). In corn, seed treatment with fludioxonil negatively affected the colonization capacity of the natural soil AMF community (Castelli et al., 2014). However, other studies reported neutral or even positive effects of fludioxonil on root colonization under greenhouse and field conditions (Hardy et al., 2025). In our study, fludioxonil strongly affected arbuscule colonization and the functionality of *R. irregularis* at concentrations ≥ 0.39 mg/L in the sandwich test. Additionally, *R. irregularis* spore germination was inhibited by fludioxonil at concentrations ≥ 0.039 mg/L. Collectively these results suggest that fludioxonil strongly affects AMF *R. irregularis* at both asymbiotic and symbiotic stages with the effects being more pronounced in the former.

4.2. *Differences in pesticide toxicity on AMF: formulation vs active ingredient*

We further explored the potential variation in the toxicity of pyraclostrobin and fludioxonil, two of the most toxic compounds in our AMF-sandwich test, depending on their form applied: commercial formulation vs pure a.i. Pesticide formulations contain additives that might alter the ecotoxicity profile of the a.i. This could take the form of increasing toxicity, in cases where formulation additives carry themselves toxicity to non-target organisms or when they facilitate the penetration of the a.i. to target and non-target organisms (Nagy et al., 2020). Alternatively, they could decrease the toxicity of the a.i., in cases where additives enhance the selectivity of the a.i. towards the target organism (Bao et al., 2022). Such comparative studies are rare for AMF. The sole example is the study of Buysens et al. (2015) who tested the effects of azoxystrobin, flutolanil and pencycuron, in the form of pure a.i. and commercial formulations, on *in vitro* spore germination, root colonization, extraradical mycelium development and spore production.

We showed that in the form of Comet®, pyraclostrobin exhibited equivalent toxicity to AMF arbuscular colonization with their EC_{50} values derived by the two different forms of pyraclostrobin being within the same range ($EC_{50\ Comet}$ 2.54 mg/L, $EC_{50\ a.i}$ 9.22 mg/L). However, at the functional level, Comet® showed a lower impact on gene expression levels and P uptake compared to the pure a.i., suggesting a reduction in the potential toxicity of pyraclostrobin by the formulation, whose underlying mechanism should be further investigated. Regardless of the form tested (a.i. or formulation), pyraclostrobin impaired *R. irregularis* at concentration levels that are within the range of concentrations expected to be found in the soil solution upon application of the recommended dose rate (3.08 mg/L), denoting a likely risk for the soil AMF community by the application of this fungicide. Conversely, fludioxonil, when used as a pure a.i., showed enhanced toxicity on *R. irregularis* arbuscular colonization compared to Scholar® ($EC_{50\ a.i}$ 0.085 mg/L vs $EC_{50\ Scolar}$ 0.58 mg/L). However, no major differences between formulation and a.i. were observed on AMF functioning, as depicted by the measurements of P uptake and expression of marker genes. Interestingly, fludioxonil, in both tested forms showed adverse effects on *R. irregularis* at concentrations which were lower than the concentration expected to be found in the soil solution upon application of the recommended dose rate (3.85 mg/L), suggesting a likely risk for the soil AMF community. Conversely monitoring studies in agricultural soils in EU have detected fludioxonil at lower concentrations of 0.02–0.075 mg/L (Hagner et al., 2024) and 2 mg/L (Bermúdez-Couso et al., 2007) (corresponding to measured soil concentrations of 0.004–0.015 mg/kg and 0.4 mg/kg, respectively). Overall, our findings suggest that formulated products might alter the ecotoxicity of pesticides on AMF. However, the direction of this change (negative or positive) could vary depending on the endpoint measured and the pesticide itself. Different pesticide formulations contain different co-formulants whose identity is often non-disclosed making difficult the establishment of clear cause-effect associations between specific co-formulants and the effects observed.

5. Conclusions

We assessed the toxicity of five different pesticides and one transformation product on the symbiotic phase of *R. irregularis* using the AMF sandwich testing system. Based on the derived EC_{50} values, the toxicity of pesticides decreased in the following order fludioxonil> glyphosate> pyraclostrobin> etridiazole > hymexazol = AMPA. The high toxicity of fludioxonil and the limited toxicity of AMPA on *R. irregularis* was also evident for the asymbiotic phase using a high-throughput spore germination assay. Overall, our study verified the potential of the gnotobiotic AMF-sandwich testing system to be used for the assessment of the toxicity of pesticides on the symbiotic phase of AMF. We showed that the test provides a broader range of toxicity endpoints that allowed us to identify a functional impairment of the symbiosis that was not evident based on the mycorrhizal colonization measurements. When used in combination with the high-throughput spore germination test, which provide complementary estimates of the toxicity of pesticides on the asymbiotic phase, the AMF-sandwich test holds promise for providing an accurate estimate of the toxicity of pesticides on AMF at Tier I level. Our findings are expected to have serious implications for the pesticide regulatory framework since they are directly relevant to the request of EFSA for new testing methods that will allow the accurate determination of the toxicity of pesticides on AMF, identified by EFSA as good proxies for assessing pesticides toxicity on the soil microbiota (EFSA Panel on Plant Protection Products and their Residues PPR et al., 2023). Inclusion of additional AMF species with different ecophysiological characteristics compared to *R. irregularis* or AMF synthetic communities in future studies with the AMF-sandwich test should be explored to enhance its broader applicability across diverse AMF species.

CRediT authorship contribution statement

Christos Papadopoulos: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Marjan Roshanfekrrad:** Investigation, Writing - Review & Editing. **Daniela Tsikou:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Kalliope K. Papadopoulou:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Maryline Calonne-Salmon:** Supervision, Methodology, Writing - Review & Editing. **Stephan Declerck:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Dimitrios G. Karpouzas:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dimitrios G. Karpouzas reports financial support was provided by Hellenic Foundation of Research and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118892.

Data Availability

Data will be made available on request.

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