



Bioremediation of Pendimethalin by the Saprophytic Fungi *Coriolopsis rigida* and *Fusarium lateritium* Rescues Symbiotic Abilities of *Phaseolus vulgaris* and Increases Soil Microbial Activities

Mario Paniagua-López^{1,2} · Lucía Martín-Lorenzo^{1,2} · Gloria A. Silva-Castro² · José A. Siles³ · Alain Valzano-Held⁴ · Francisco Palma¹ · Inmaculada García Romera² · Miguel López-Gómez¹

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Abstract

The increment in the use of herbicides in the last decades has reduced the soil microbial diversity affecting the symbiotic associations between legumes and soil microorganisms, which are essential for plant nutrition and therefore agricultural production. Consequently, in this work, we have evaluated the potential of two saprophytic fungi as a bioremediation tool for the recovery of soil microbial activity and the symbiotic abilities in *Phaseolus vulgaris*. Initially, the saprophytic fungi *Coriolopsis rigida* and *Fusarium lateritium* were tested for their capacity to degrade pendimethalin in vitro. After that, the ability of both fungi to improve the symbiotic capacity of *Phaseolus vulgaris* was assessed in a pot trial in which parameters related to plant growth, nitrogen fixation and arbuscular mycorrhizal fungi (AMF) root colonization were analysed together with the soil microbial activity. We observed that *C. rigida* and *F. lateritium* utilized pendimethalin as a carbon source and mitigated the plant biomass reduction as well as the inhibition of nitrogen fixation and AMF root colonization provoked by the herbicide in *P. vulgaris*. Additionally, the inoculation with both fungi led to increased microbial activities in the soil sprayed with herbicide, which was associated with the dissipation of pendimethalin induced by both fungi in inoculated soils. The results confirmed the effectiveness of *C. rigida* and *F. lateritium* as bioremediation tool for the removal of pendimethalin to improve soil microbial activity and the symbiotic interactions between soil microbes and *P. vulgaris*. These findings contribute to the development of sustainable agricultural practices.

✉ Mario Paniagua-López
mario.paniagua@eez.csic.es

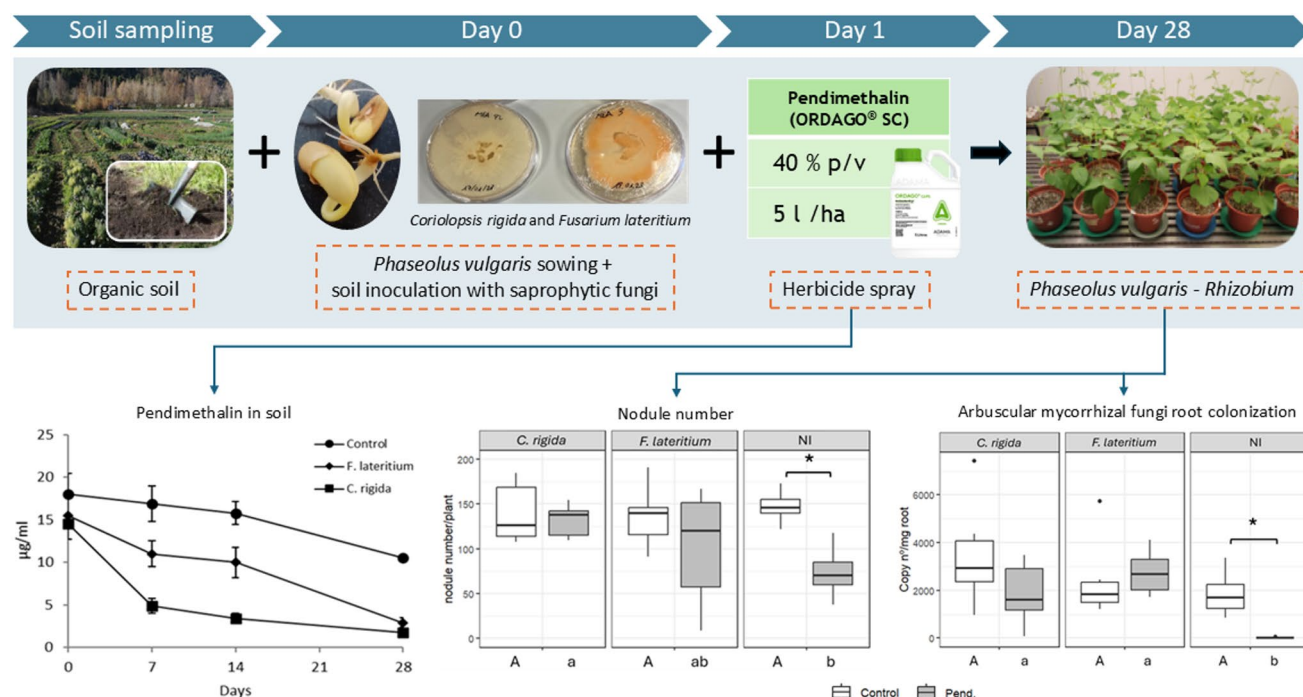
¹ Department of Plant Physiology, Faculty of Sciences, University of Granada, Campus de Fuentenueva, Granada 18071, Spain

² Department of Soil and Plant Microbiology, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (EEZ-CSIC), C/Profesor Albareda, 1, Granada 18008, Spain

³ Department of Soil and Water Conservation and Organic Waste Management, CEBAS-CSIC, Murcia 30100, Spain

⁴ Department of Plant and Microbial Biology, University of Zurich, Zollikerstrasse 107, Zurich 8008, Switzerland

Graphical Abstract



Keywords Bioremediation · Pendimethalin · Symbiosis · Legumes · *Coriopsis rigida* · *Fusarium lateritium*

1 Introduction

Pesticides are fundamental part of current agriculture and contribute to the control of weeds, plant diseases, and pests. Over the past two decades, the use of pesticides has increased over 40%, and currently, over 4.1 million tons are used worldwide every year (FAO 2024). Depending on their application, only a minor fraction of the applied pesticides reaches their targets (Rodríguez-Eugenio et al. 2018), with the rest (30 to 50%) ending up on the soil surface. The excessive and massive use of pesticides is one of the factors with a higher impact on the whole ecosystem and particularly on soil microorganisms such as nitrogen fixing bacteria and arbuscular mycorrhizal fungi (AMF), which drive crucial soil processes such as carbon and nutrient cycling (Van Der Heijden et al. 2016), essential for plant nutrition and therefore agricultural production (Bünemann et al. 2006). Indeed, studies with individual pesticides on specific microorganisms have shown that specific pesticides constitute a threat to soil microorganisms (Bünemann et al. 2018).

Among the pesticides used worldwide, herbicides comprise about 50% of the total (Aloo et al. 2021; FAO 2024) and have been shown to specifically affect the soil microbial diversity and the symbiotic associations between plants and soil microorganisms, including nitrogen fixing

bacteria (Burul et al. 2022; Fox et al. 2007). Pendimethalin is, after glyphosate and paraquat, the third most used herbicide as pre-emergence for the control of annual weeds in a broad variety of crops (Mohamed and Abdalla 2023), including leguminous such as peas (*Pisum sativum* L.) and beans (*Phaseolus vulgaris*). Pendimethalin is indicated for direct application on the soil, which can provoke its accumulation in agricultural fields (Kjær et al. 2011; Luks et al. 2021) with higher risk of affecting non target organism. In fact, field dissipation studies have shown that pendimethalin is relatively stable in soil and resistant to degradation (Kulshrestha et al. 2000; Strandberg and Scott-Fordsmand 2004), and therefore, can inhibit legume-rhizobia symbiosis (Bollich et al. 1988) including the *P. vulgaris*-*R. tropici* interaction (Paniagua-López et al. 2023) and AMF symbiosis, with different sensibilities depending on the fungal strains (Hage-Ahmed et al. 2019). Preventing the negative influence of pendimethalin in these beneficial plant microbe interactions (e.g. reducing survival and growth of rhizobia, inhibiting nodulation process, and depleting nitrogen fixation) (Eberbach 2018) is an environmental challenge that needs to be addressed. This is of great importance for the positive effect of these symbiosis on soil fertility by means of its capacity to fix atmospheric nitrogen at the plants root level (Coba De La Peña and Pueyo 2012). In fact, the vast majority of biologically fixed

N is attributable to symbioses between leguminous plants (soybean, alfalfa, etc.) and species of *Rhizobium* bacteria (Peoples et al. 2021).

Given the concern about soil pollution by pesticides, several biological methods for pesticide removal have been developed and widely implemented to protect soil health and restore soil properties (Raffa and Chiampo 2021). Among these biological methods is bioremediation, which represents an efficient, cost-effective, and environment-friendly technique that involves accelerating the rate of natural degradation of organic pollutants through plants, bacteria, fungi or yeast preparations (Alegbeleye et al. 2017; Szyrka et al. 2024). In the bioremediation mediated by fungi, the pesticides are directly used as a carbon or nutrient sources and sometimes, extracellular enzymes are secreted to transform the toxic pesticide into non-toxic molecules. These enzymes include oxidoreductases (laccase, peroxidase, oxygenase), hydrolases (cellulase, protease) and transferases. The pesticide degradation process occurs in three steps consisting in the bioadsorption, in which the pollutants get adsorbed on the microbial surface, then through absorption accumulated inside the cell, and in the third step, the pesticide molecules are degraded through multiple enzymatic activities (Chaudhari et al. 2023). White-rot fungi are one of the main producers of oxidative enzymes for the remediation of polluted environments. These microorganisms are very effective due to their robustness and capacity to tolerate high concentration of pollutants (Pinto et al. 2012). Saprophytic fungi from the *Coriopsis* and *Fusarium* genera have been used for the removal of phenols from agro-industrial waste (Arriagada et al. 2009; Sampedro et al. 2004), as well as in the degradation and detoxification of phenanthrene (PHE) and recalcitrant dyes (Agrawal et al. 2022; Chen and Yien Ting 2015). The ability of *Coriopsis rigida* to degrade lignin, sterols, and several hazardous pollutants like polycyclic aromatic hydrocarbons, has been described (Saparrat et al. 2014). Additionally, *C. rigida* in association with AMF appears to enhance plant growth in heavy metal contaminated soil (Arriagada et al. 2010). Similarly, the white-rot fungi *Fusarium oxysporum* and *F. solani* have demonstrated the ability to degrade pesticides such as pendimethalin (Barua et al. 1990; Kulshrestha et al. 2000), emphasizing their potential for bioremediation.

Therefore, we hypothesized that soil inoculation with *C. rigida* and *F. lateritium* would reduce the persistence of pendimethalin in the soil, which would improve the soil biological activity and hence, the symbiotic performance of *P. vulgaris* plants. In order to test this hypothesis, in this work we have conducted a pot trial in which parameters related to plant growth and symbiotic response has been analysed together with soil biological activity.

2 Materials and methods

2.1 Fungal Cultures

Two different saprobic fungal species with potential ability to tolerate and degrade pesticides by using them as carbon source (Reyes-César et al. 2014; Daccò et al. 2020) were selected for this study from the fungal collection of the Estación Experimental del Zaidín (EEZ-CSIC): *Fusarium lateritium* (EEZ-3), and *Coriopsis rigida* (EEZ-92).

2.2 Culture in Liquid

To prepare the initial fungal inoculum, 250 ml flasks containing 70 ml of Czapek solution (pH 6.5) were each inoculated with two 1 cm³ cubes of precultured fungi. The flasks were incubated for one week under constant agitation and total darkness (80 rpm; 28 °C). After growth, the mycelia were washed, filtered, and homogenized in sterile water. Subsequently, 1.5 ml of this fungal inoculum suspension were added to 250 ml flasks containing 50 ml of Czapek and supplemented either with pendimethalin (33 mg l⁻¹; equivalent to 3 l ha⁻¹, the lowest value of the recommended dosage range of the product) or sucrose (30 g l⁻¹) as sole carbon source. Each fungal culture was carried out in triplicate, incubated under the conditions described above, and sampled over time (0, 4, 7, 14, and 21 days). Furthermore, control samples without fungal inoculum were incubated to measure pendimethalin abiotic degradation.

At each sampling time the fungal mycelium was filtered, dried, and weighted and the concentration of pendimethalin was measured both in the liquid media and in the fungal mycelia (both adsorbed on the biomass surface and absorbed in the mycelia) following an extraction using 90% cold acetone and further separation with hexane. The upper nonpolar hexane phase containing the extracted pendimethalin was collected and an aliquot of 1 ml was brought to dryness under air flow and kept freeze in N₂ atmosphere until measurement in HPLC (Agilent 1260 Infinity). To determine the proportion of pendimethalin removal from the liquid culture attributed to adsorption and absorption by the fungi, the pendimethalin retained in the fungal biomass was separated into two fractions. The first fraction was obtained by washing the mycelia following the methodology described below for herbicide extraction from the culture media. The extracted fraction of pendimethalin in this step was considered the proportion retained by the fungi by adsorption. For the obtention of the second fraction, the previously washed fungal biomass was finely ground manually using a porcelain mortar and the pendimethalin contained in this material was extracted following the same procedure.

The extracted fraction of pendimethalin in this step was considered the proportion absorbed by the mycelium.

2.3 Culture in Solid Media and Inoculation

For fungal inoculation of plants, a 1 cm³ cube of precultured fungi was first cultured on 2% malt extract agar plates for one week at 28 °C to maintain a fresh inoculum. Subsequently, the fresh fungi inoculum was cultured in barley-based media (18 g of sterile barley seeds and 30 ml of sterile water) by adding 10 ml of the homogenized content of a fungal agar plate in 40 mL of sterile water. After a period of two weeks of incubation, the soil inoculation was performed by adding 10 g of the homogenized barley-based media colonized by the fungus to each experimental pot, mixed thoroughly with the soil prior to seedling transplantation. Non-inoculated plants were supplied with the same amount of sterile barley seed as inoculated plants.

2.4 Plant Material and Growth Conditions

Phaseolus vulgaris (var. Efe15) seeds were surface sterilized by immersion in 5% NaClO plus Tween 20 (Sigma-Aldrich, Saint Louis, MI USA) for 5 min and germinated onto wet sterilized filter paper at 28° C in the darkness. After 3 days, 2 seedlings were transferred to individual pots of 1.8 l filled with a soil: perlite mixture (3:1, v/v). The experiment followed a factorial design with two herbicide conditions (control and pendimethalin) and three inoculation treatments: non-inoculated (NI), inoculated with *C. rigida*, or inoculated with *F. lateritium*. This resulted in six treatment combinations, each with 12 biological replicates (one plant per pot). Pendimethalin was applied 1 day after sowing as a pre-emergence herbicide according to the conditions recommended by the manufacturer and considering variables such as phenological stage of the plants and soil moisture. The commercial product Ordago® (ADAMA Ltd.®), with pendimethalin (cycloexanodiona, HRAC) as active ingredient (AI), was applied following the recommended label dosage detailed in Table 1, and following the procedure described in Paniagua-López et al. (2023).

The pots were watered twice a week to keep 65% of soil water holding capacity (WHC) and grown in a controlled environmental chamber with a 16/8 h light–dark cycle, 26/22°C day night temperature, relative humidity 55/75% and photosynthetic photon flux density (400–700 nm) of 450 l mol m⁻² s⁻¹. Four weeks after sowing, when the plants were at vegetative growth stage and the symbiosis was well established, root nodules were detached, quantified and weighed to obtain nodule number (NN) and nodule fresh weight (NFW). Shoots and roots were dried at 70 °C for 24 h to determine shoot dry weight (SDW) and root dry weight (RDW).

Table 1 Detailed information on the herbicide product used

Herbicide	Classification HRAC*	Product name	Manufacturer	Concentration active ingredient (AI)	Product dosage	Product amount per pot
Pendimethalin	Cycloexanodiona	Ordago®	ADAMA (Spain)	40% p/v	3 l ha ⁻¹ (1.2 kg AI ha ⁻¹)	3,07 µg

*HRAC: International herbicide-resistant weed database (<http://www.weedscience.org/Pages/aboutus.aspx>)

2.5 Nitrogen Fixation

Nitrogenase activity (E.C. 1.7.9.92) was measured as the representative H_2 -evolution in an open-flow system (Witty and Minchin 1998) using an electrochemical H_2 sensor (Qubit System Inc., Canada). H_2 production was recorded on 0.2 g of representative nodulated roots sample immediately after plant harvest. Apparent nitrogenase activity (ANA, rate of H_2 production in air) was determined under N_2/O_2 (80:20%) with a total flow of 0.4 l min^{-1} . After reaching steady-state conditions, total nitrogenase activity (TNA) was determined under Ar/O_2 (79:21%). Nitrogen fixation rate (NFR) was calculated as $(TNA-ANA)/3$. Standards of high purity H_2 were used to calibrate the detector.

2.6 Arbuscular Mycorrhizal Fungi Root Colonization

Arbuscular mycorrhizal fungi (AMF) root colonization was initially evaluated by the microscopy-based magnified intersection method described by (McGonigle et al. 1990). Afterwards, AMF root colonization was quantified by the qPCR method described by Bodenhausen et al. (2021) with some modifications. First, the frozen root samples were lyophilized for 24 h and then grounded using a FastPrep (MP Biomedicals™). DNA was isolated from 25 mg of the grounded roots using the MN-Plant II 96-well kit (Macherey204 Nagel, item no.: 740663). The extraction was performed according to the manufacturer's protocol. DNA quantification was carried out using the Lumiprobe DNA quantification kit (Lumiprobe Pico488 dsDNA quantification kit, item no.: 1102–200) with a Varian Plate Reader (Varian Cary Eclipse Fluorescence Spectrophotometer, EL02015507). The DNA was then diluted to a concentration of $5\text{ ng }\mu\text{l}^{-1}$ using a pipetting robot (Gilson Pipetmax 32100000). The primers AMG1 (5'-gttcccgtaagcgccgaa-3') and AM1 (5'-atagggatagttggggcat-3') were used for the absolute quantification of total mycorrhization. For each qPCR run, a standard curve was measured using a dilution series of the qPCR standard to determine the gene copies per ng of DNA. The qPCR Standard was synthesized as a double-stranded gen fragment using the reference sequence from the NIH Gen Bank (LN609699). Each $10\text{ }\mu\text{l}$ qPCR reaction was performed with $2\text{ }\mu\text{l}$ HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne item no.: 08–25–00020), $0.5\text{ }\mu\text{l}$ of each primer ($10\text{ }\mu\text{M}$) and 25 ng of root DNA. qPCR reactions were performed in triplicates on a CFX Opus 384 (CFX Opus 384 Bio Rad) in a 384-well plate. The qPCR cycle consisted of a polymerase activation step of 12 min at $95\text{ }^\circ\text{C}$, followed by 40 cycles of DNA denaturation at $95\text{ }^\circ\text{C}$ for 15 s, primer hybridisation (annealing) at $62\text{ }^\circ\text{C}$ for 30 s, elongation at $72\text{ }^\circ\text{C}$ for 20 s, followed by a melting curve (from 65 to $95\text{ }^\circ\text{C}$ at $0.5\text{ }^\circ\text{C}/5\text{ s}$). The raw data was exported

from Bio-Rad CFX Maestro as an Excel file to LinReg to determine the cycle count (Ct) from the baseline calculated by LinReg.

2.7 Soil Characterization

The soil used was collected from an organic production farm near Granada, Spain (N $37^\circ 9'42.42.9''$ W $3^\circ 33'23.889''$) in January 2024. The soil was taken from the first 15 cm of the surface of a previously fallow plot. Soil was air dried at room temperature in the laboratory, sieved at 2-mm, and homogenized. This fraction was used to determine the main physico-chemical properties of the soil following standard methods (MAPA 1994), shown in detail in Table S1. Soil: water extract was prepared to determine the pH (1:2.5) and electrical conductivity (EC) (1:5). Soil texture was calculated by the Robinson pipette method (Loveland and Whalley 1991) calcium carbonate content ($CaCO_3$) was measured by manometric method according to Barahona (1984) and organic carbon (OC) was determined according to Tyurin (1951). Total nitrogen (N) and carbon (C) were analysed by dry combustion using an elemental analyser TruSpec CN LECO® (St. Joseph, MI, USA). Available phosphorous (Pav) was analysed according to Olsen and Sommers (1982).

Soil biological activity parameters such as enzymatic activities and soil basal respiration were also measured. The basal respiration rate (ISO 2002), based on CO_2 emission by soil microorganisms, was determined by indirect impedance measurement with a BacTrac 4200 Microbiological Analyser (SY-LAB, Austria). Before starting the test, soil moisture was adjusted at water holding capacity. Urease was measured as described by Kandeler et al. (1999). Phosphatase and β -glucosidase activities were assessed following the methods described by Eivazi and Tabatabai (1977, 1988), respectively.

2.8 Pendimethalin Quantification

For pendimethalin quantification in the liquid cultures of each fungus (both in liquid media and in mycelia) the complete sample obtained at each sampling time was used. In soil samples, pendimethalin was quantified using 5 g of soil surface from each treatment. The herbicide was extracted from the different samples using 20 ml of 90% cold acetone. Soil samples were first shaken for 2 h in darkness, and then centrifuged at $12,000\text{ g}$ and $4\text{ }^\circ\text{C}$ during 10 min. The supernatant was diluted with 5% NaCl in water and partitioned with n-hexane at 1:4 (v/v). The upper nonpolar hexane phase containing the extracted pendimethalin was collected, and an aliquot of 1 ml was evaporated to dryness under air flow. The samples were kept frozen in N_2 atmosphere and redissolved in 1 ml acetonitrile prior to measurement.

The concentration of pendimethalin was determined using an HPLC instrument (Agilent 1260 Infinity) with a Zorbax Eclipse-AAA 4.6 mm×150 mm column and a diode array at 255 nm. The herbicide was eluted at a flow rate of 1.2 ml min⁻¹ using an elution gradient with acetonitrile (A) and 0.1% formic acid in Milli-Q water (B). The gradient profile, expressed as (t [min]; %A), was: (0; 60%), (1; 60%), (7; 100%), (12; 100%), and (13; 60%). Finally, the column was regenerated for 4 min after each run.

2.9 Abundance of Bacteria and Fungi in Soil

Analysis of the soil contents of ester-linked fatty acid methyl esters (EL-FAME) was used to assess the abundance of bacteria and fungi. EL-FAME contents were measured following the method developed by Schutter and Dick (2000). Briefly, fatty acids were extracted from microbial cells and released as methyl esters by incubating 3 g of frozen soil with 15 ml 0.2 M methanolic KOH during 1 h at 37 °C under periodic shaking. Samples underwent pH neutralization with 1 M acetic acid. Subsequently, EL-FAMES were partitioned into an organic phase by adding 10 ml hexane and vigorous shaking, followed by centrifugation and evaporation of the hexane in a SpeedVac (Labogene). EL-FAMES were finally resuspended in iso-octane and analysed by gas chromatography under the chromatographic conditions described by Siles et al. (2024). Soil bacterial biomass was quantified by summing the amounts of the fatty acids i15:0, a15:0, i16:0, 16:1ω9c, 10Me16:0, i17:0, cy17:0, 10Me18:0, and cy19:0. For fungal biomass, the amounts of the fatty acids 18:2ω6,9t and 18:2ω6,9c were summed (Joergensen 2022). Data were expressed as EL-FAME g⁻¹ dry weight soil. The relative abundance of the unspecific microbial fatty acids 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0, along with those of the bacterial and fungal markers, were used to assess variations in the structure of the microbial community between treatments.

2.10 Statistical Analysis

Experiments were arranged in a completely randomized design with 12 plants per treatment in the pot trial. The data were subjected to a two-way analysis of variance (ANOVA) analysis with herbicide treatment (untreated control, or pendimethalin) and inoculation with fungi as the variable factors using the IBM SPSS Statistics 28.0.0.0 software and significant differences among treatments were determined by Tukey's post-hoc test ($P \leq 0.05$). Levene's test was used to check homogeneity of variances. Mean values ± SE error bars are represented. NMDS (non-metric multidimensional scaling) analysis based on Bray-Curtis dissimilarities was

used as ordination method to visualize changes in the fatty-acid based structure of soil microbial communities taking advantage of the metaMDS function in the R package vegan ver. 2.6–4. Significance of the changes in the structure of microbial community was tested by PERMANOVA (permutational analysis of variance) with Bray-Curtis dissimilarities after 9999 per-mutations using the R package vegan ver. 2.6–4. Principal component analysis (PCA) was performed to determine the relationships between the treatments and the main variables studied both at soil and plant level using the software Statgraphics Centurion Version 19.6.03.

3 Results

3.1 Fungal Growth in Liquid Culture

Coriopsis rigida and *Fusarium lateritium* showed great capacity to grow in the liquid minimum media containing only pendimethalin as a carbon source, as evidenced by the increase in mycelium biomass (Fig. S1). The biomass increments during the first week were similar in the media containing pendimethalin and sucrose as carbon source. However, after two, and more evidently after three weeks, a reduction in the fungal biomass with pendimethalin as carbon source can be observed with respect to sucrose. In the case of *F. lateritium*, this reduction was about 20%, while in *C. rigida*, the difference was 33% lower biomass production related to the sucrose. Despite these differences, the growth tendencies shown over time by the two fungi were similar, although the rate of biomass production of *F. lateritium* was about 10-fold higher when compared to *C. rigida*. Concomitant with the increase in fungal biomass, a dramatic reduction of the pendimethalin concentration in the liquid culture media was detected (Fig. 1). The evolution of pendimethalin removal rate from the liquid culture shows that the highest removal rate occurred in the first days of exposure, with *F. lateritium* achieving a near complete removal rate (>99%) after 3 days. In comparison, *C. rigida* showed a slower removal rate, reaching removal percentages of >99% after 7 days. By the end of the assay, both fungi had achieved 100% removal, in contrast to about 38% of pendimethalin degradation occurred in the control samples, indicating pendimethalin abiotic degradation rate. The reduction in pendimethalin concentration in the liquid culture in the presence of the fungi was accompanied by an increment in the pendimethalin adsorbed onto the mycelium, especially by *F. lateritium*. Additionally, pendimethalin absorption into the mycelium occurred at similar rates in both fungi, despite the differences in fungal biomass present in the culture.

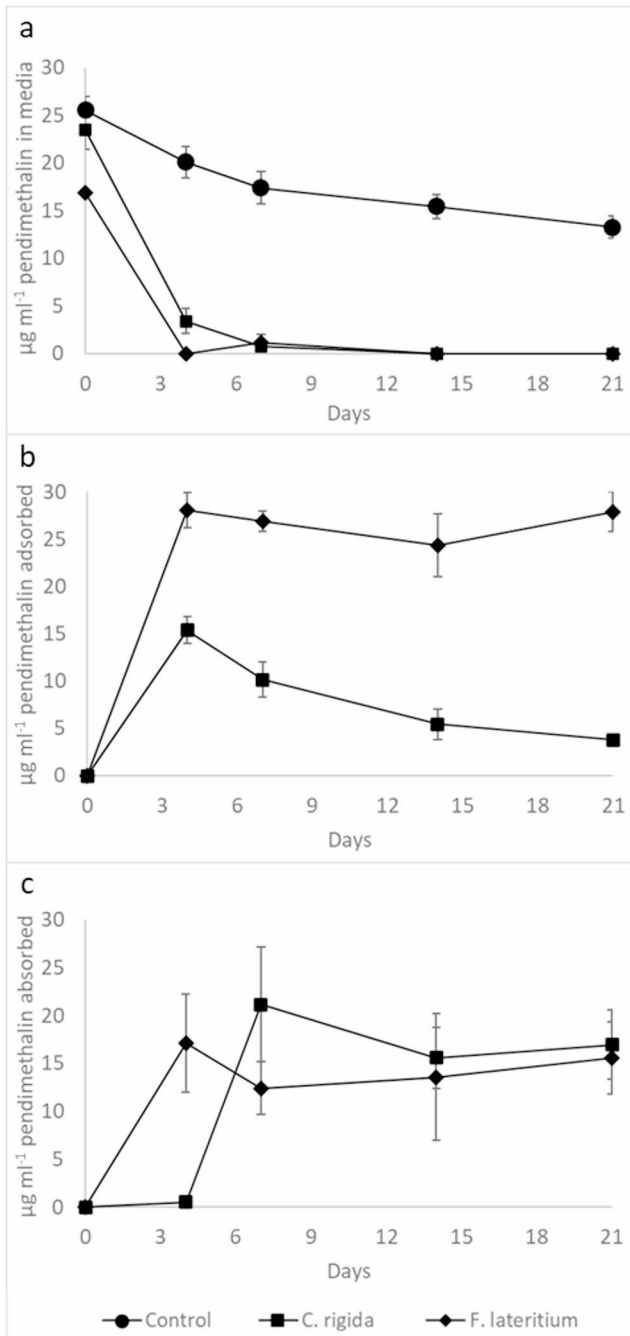


Fig. 1 (a) Removal kinetics of pendimethalin in culture media in not inoculated (Control) and inoculated with *C. rigida* and *F. lateritium* treatments; (b) Concentration of pendimethalin adsorbed to the mycelium of *C. rigida* and *F. lateritium*; (c) Concentration of pendimethalin absorbed by the mycelium of *C. rigida* and *F. lateritium*. Data represent mean $\pm$ SD ($n=3$)

3.2 Plant Growth

Herbicide spraying caused a significant reduction of 30% in total plant biomass, affecting both shoot and root dry weight. Shoot dry weight decreased by 25% while root dry

weight was particularly impacted, exhibiting only half the root biomass of control plants (Fig. 2). Inoculation with the two saprophytic fungi mitigated the plant biomass reduction provoked by the herbicide, except for the root dry weight in plants inoculated with *F. lateritium*, in which a similar reduction to non-inoculated plants in the presence of the herbicide was observed. Under control conditions, the inoculation with both fungi had no significant effect on total plant dry weight, except for shoot biomass, which increased by 30% with *C. rigida* compared to control plants.

3.3 Nitrogen Fixation and AMF Colonization

Nitrogen fixation parameters (Fig. 3), including nodule number and biomass, were strongly reduced by the herbicide effect, showing half the values regarding the control conditions. Nitrogen fixation rate was also inhibited by the herbicide about 40%. However, inoculation with the saprophytic fungi mitigated the negative effect of the herbicide treatment in all parameters, with no significant differences between control and sprayed plants. This effect was especially evident in the plants inoculated with *C. rigida*, which showed a significant increment in the nodule number and biomass with respect to non-inoculated plants under the herbicide treatment. Interestingly, *F. lateritium* provoked a significant reduction in the nodule biomass in control conditions that did not affect the nitrogen fixation rate per gram of nodule.

A similar pattern was observed for AMF root colonization (Fig. 4), that showed a dramatic reduction by the herbicide in non-inoculated plants, while not significant differences between control and herbicide treated plants were detected due to the increment induced by the fungal inoculation.

3.4 Pendimethalin Evolution in Soil

The concentration of pendimethalin in the soil was monitored weekly to evaluate the effect of fungal inoculation on herbicide dissipation in the first cm of soil (Fig. 5). Seven days after spraying, the dissipation rate of pendimethalin was $1.36 \mu\text{g day}^{-1}$ and $0.64 \mu\text{g day}^{-1}$ in soil inoculated with *C. rigida* and *F. lateritium*, respectively, while in not inoculated soil, the dissipation rate was $0.14 \mu\text{g day}^{-1}$. By day 28, the total dissipation of pendimethalin in soils inoculated with *C. rigida* and *F. lateritium* was 88%, and 81%, respectively, whereas in the control the dissipation was only 42%. This indicates that fungal inoculation doubled the pendimethalin dissipation rate.

3.5 Soil Biological Activity

Soil biological activity was assessed by determining key enzyme activities involved in nutrient cycling and through soil respiration (Table 2). β -glucosidase activity was

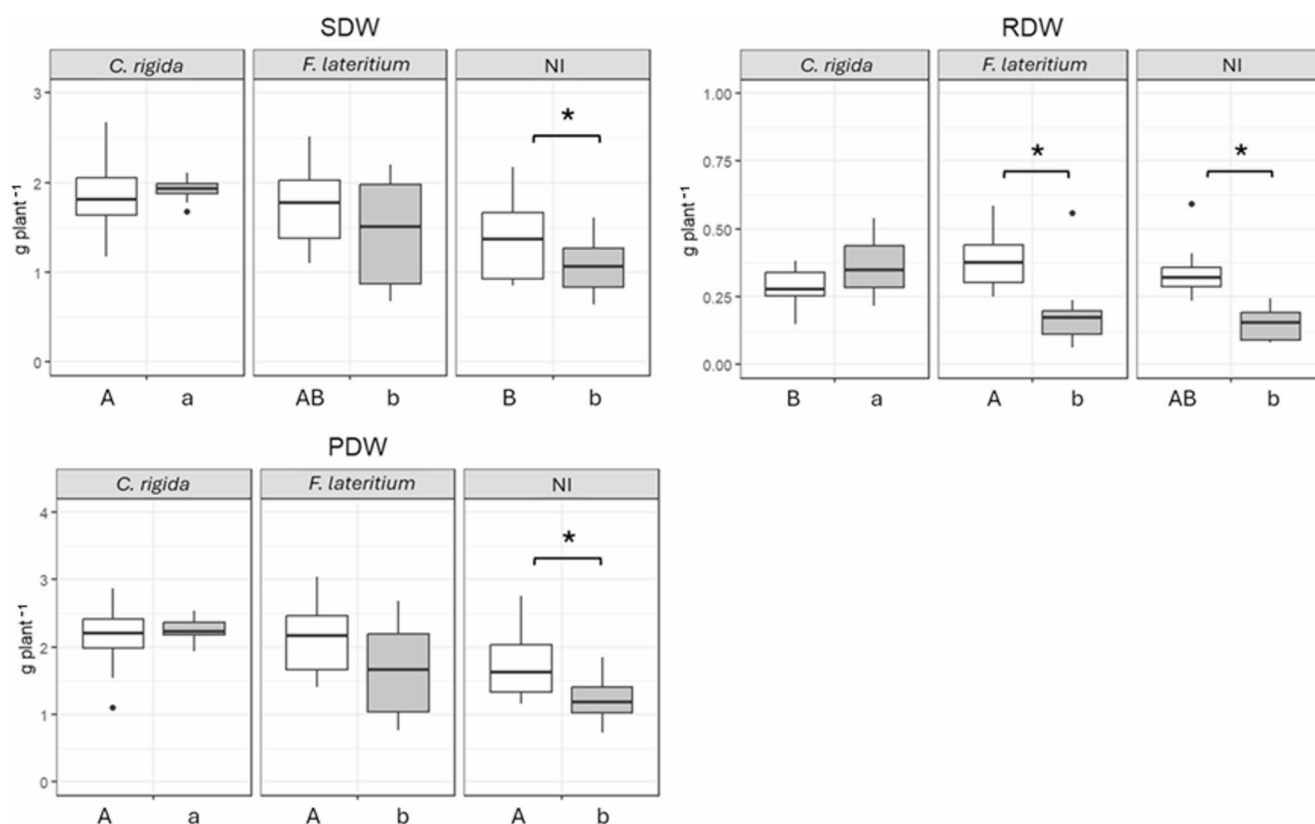


Fig. 2 Shoot dry weight (SDW), root dry weight (RDW), and plant dry weight (PDW) of *P. vulgaris* not inoculated (NI) and inoculated with *C. rigida* and *F. lateritium* under control (white boxes) and pendimethalin (grey boxes) treatment. Capital letters

indicate statistically different groups among control and lowercase letters statistically different groups among pendimethalin treatment according to Tukey's HSD test. Asterisk (*) denotes significantly different values Fisher's LSD test $p < 0.05$

reduced by 30% in the presence of pendimethalin in non-inoculated soils, whereas inoculation with *C. rigida* and *F. lateritium* mitigated the negative effect of the herbicide on this enzymatic process. However, fungal inoculation did not prevent the 30% inhibition of phosphatase activity by pendimethalin. Interestingly, B-glucosidase and phosphatase activities showed a significant reduction in *F. lateritium* inoculated soils, namely, 45% and 35%, respectively, while in pendimethalin-sprayed soils, phosphatase activity increased by 30% with *F. lateritium* inoculation. Urease activity and basal soil respiration remained unaffected by fungal inoculation and/or herbicide application.

3.6 Microbial Abundance and Community Structure

The presence of herbicide in the soil did not impact the abundance of bacteria and fungi (Fig. 6). However, the inoculation with *C. rigida* and *F. lateritium* led to increased amounts of both bacteria (ANOVA, $p < 0.0001$) and fungi (ANOVA, $p < 0.0001$) in the soil sprayed with herbicide, while in control conditions, this effect was only detected with *F. lateritium*. After 28 days, the fungal abundance was

higher in the samples inoculated with *F. lateritium* than in those with *C. rigida*.

PERMANOVA demonstrated that the structure of the microbial community did not change with herbicide application ($F = 2.43$, $p = 0.066$), but it changed with the soil inoculation with the fungi ($F = 12.87$, $p = 0.0001$) and the interaction between herbicide $\times$ fungal inocula ($F = 2.55$, $p = 0.023$). This was graphically demonstrated by the NMDS (Fig. S2), which evidenced that the main factor ordinating soil samples was the inoculation or not of soil with fungi and the type of fungi inoculated. Soil application of *F. lateritium* seems to alter the structure of the soil microbial community to a higher extent than *C. rigida* respect to the control soil.

3.7 Implications of Treatments at Soil and Plant Level

The principal component analysis (PCA), performed as an overall evaluation of the processes occurring under the different treatments, involving both soil and plant status, highlighted the variability of the treatments based on their influence on nodulation, nitrogen fixation rate, soil enzymatic

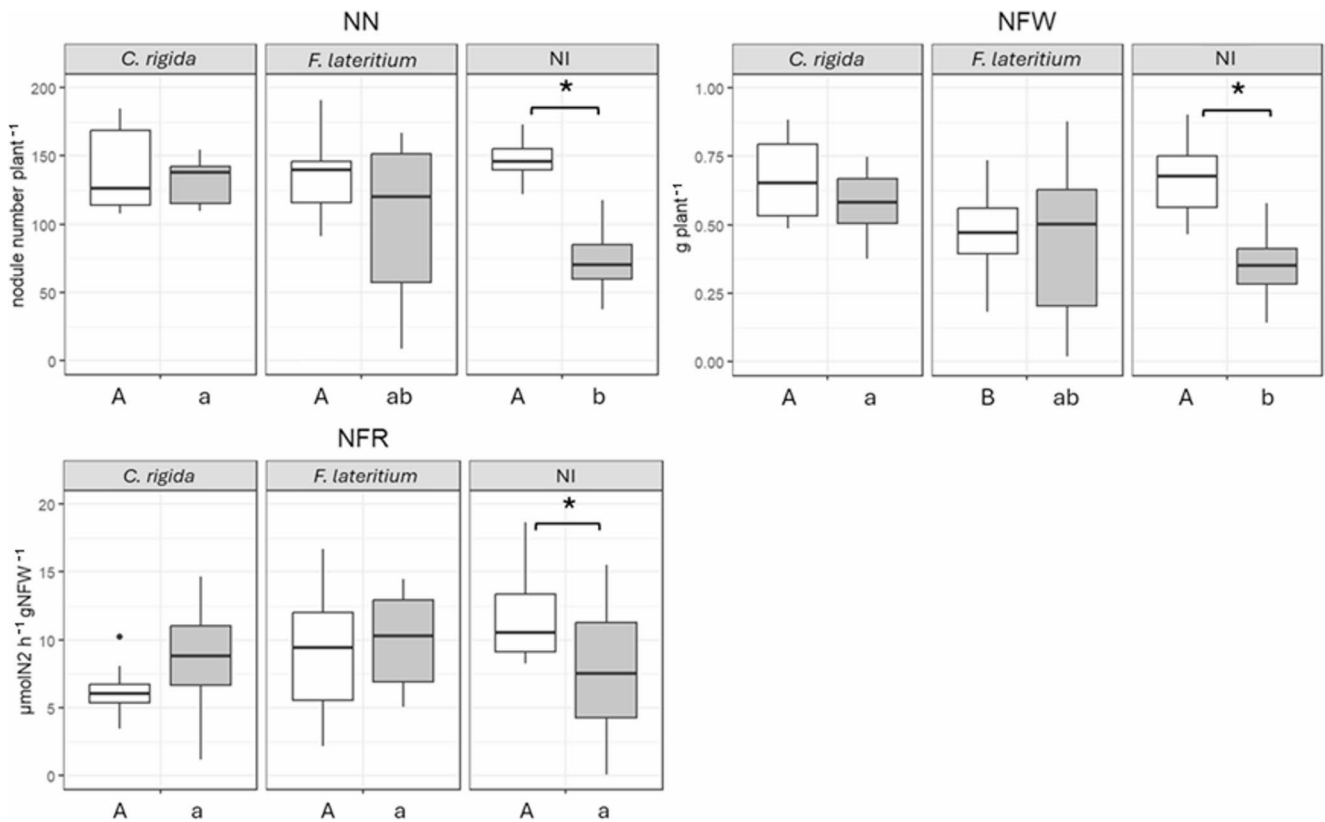


Fig. 3 Nodule number (NN), nodule fresh weight (NFW) and nitrogen fixation rate (NFR) of *P. vulgaris* not inoculated (NI) and inoculated with *C. rigida* and *F. lateritium* under control (white boxes) and pendimethalin (grey boxes) treatment. Capital letters indicate

statistically different groups among control and lowercase letters statistically different groups among pendimethalin treatment according to Tukey's HSD test. Asterisk (*) denotes significantly different values Fisher's LSD test $p < 0.05$

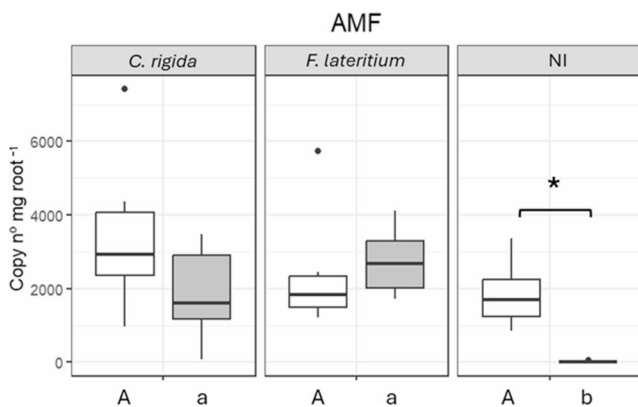


Fig. 4 Arbuscular mycorrhizal fungi (AMF) root colonization of *P. vulgaris* not inoculated (NI) and inoculated with *C. rigida* and *F. lateritium* under control (white boxes) and pendimethalin (grey boxes) treatment. Capital letters indicate statistically different groups among control and lowercase letters statistically different groups among pendimethalin treatment according to Tukey's HSD test. Asterisk (*) denotes significantly different values Fisher's LSD test $p < 0.05$

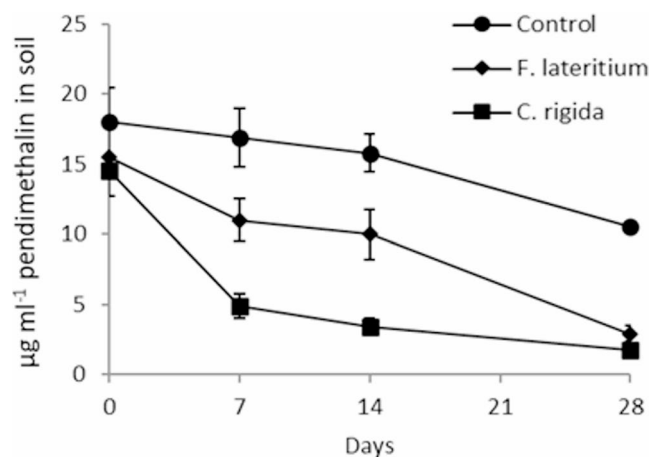


Fig. 5 Dissipation kinetic of pendimethalin in soil not inoculated (Control) and inoculated with *C. rigida* and *F. lateritium*. Data represent mean $\pm$ SD ($n = 3$)

activities, and plant growth (Fig. 7). In this regard, distinct treatment groupings were identified. Specifically, individual groups were differentiated between plants inoculated with *F. lateritium* (brown-coloured group), both in the presence and

absence of herbicide, and plants inoculated with *C. rigida* (green-coloured group), also with and without herbicide. Additionally, a group representing the herbicide-treated control (H) was clearly separated from the rest, with a significant indirect correlation with all evaluated parameters.

In this regard, previous studies have shown the resistance to pendimethalin and high capacity of mycelial growth in culture media contaminated with this herbicide of *Fusarium* spp. (Bravim et al. 2021). In addition to adsorption, absorption of the herbicide into the fungal mycelium occurred in parallel, showing both fungi similar absorption rates. Despite its lower biomass, *C. rigida* demonstrated a high ability to absorb significant rates of herbicide, suggesting an efficient mechanism for herbicide uptake even with a limited biomass production. The capacity of these fungi to immobilize and remove the herbicide from the culture, and further degrade it, may be associated with laccase production. Laccases are ligninolytic enzymes produced by fungi capable of catalytically decomposing a wide variety of harmful chemical compounds, such as herbicides (Datta et al. 2021; Singh Arora and Kumar Sharma 2010). The application of fungal laccases have been proposed as a potential strategy for the bioremediation of different pesticides for their potential to degrade them and tolerance to adverse conditions (Kumar et al. 2021; Rudakiya et al. 2020). By these mechanisms, both fungi contributed to reducing the concentration of pendimethalin in the growth liquid media to undetectable levels. This is consistent with previous studies demonstrating the ability of other fungal species, such as *F. oxysporum*, *Aspergillus orizae* and *Penicillium brevicompactum*, to degrade pendimethalin, among other herbicides, in liquid media (Bravim et al. 2020; Pinto et al. 2012).

P. vulgaris growth parameters were significantly reduced by the pendimethalin treatment (Fig. 2), which was also observed in our previous work (Paniagua-López et al. 2023). However, in this experiment, we demonstrated the ability of the two fungi tested to mitigate the negative effect of the herbicide in the plant biomass production. This beneficial effect of fungal inoculation would be related to their capacity to reduce the concentration of pendimethalin in the soil (Fig. 5). In addition to its ability to degrade pesticides, *C. rigida* has also been shown to degrade recalcitrant dyes (Chen and Yien Ting 2015).

The nodulation and nitrogen fixing capacity of *P. vulgaris* were severely reduced by pendimethalin (Fig. 3), which has also been observed in other species of legumes such as *Bituminaria bituminosa* (Mohamed et al. 2021) and chickpea (Khan et al. 2004), as well as in our previous work in *P. vulgaris* (Paniagua-López et al. 2023). The reduction in the nitrogen fixation capacity of legumes due to herbicides exposure has been associated with the inhibition of rhizobial functions in the rhizosphere (Singh 2017; Strandberg and Scott-Fordsmand 2004) and with the disturbance in the phytochemical signalling needed for the symbiont's recognition and coordination (Fox et al. 2007). Additionally, the root colonization by AMF was also severely reduced (Fig. 4). In the case of the most used

herbicide glyphosate, an effect on AMF spore germination and hyphal growth has not been shown under the recommended field concentrations (Pasaribu et al. 2011). In contrast, de Novais et al. (2019) observed that AMF growth in the presence of different herbicides, including pendimethalin, led to a reduction and disruption of AMF mycelial structural traits. Furthermore, pendimethalin has been shown to reduce AMF root colonization in corn plants (*Zea mays*) when applied to the soil (Siqueira et al. 1991) and to adversely affect spore germination and mycelial growth even at lower concentrations than those indicated for use (Giovannetti et al. 2006). The dramatic reduction in the AMF root colonization observed in the presence of pendimethalin might therefore be related to the inhibition of AMF development in roots, as well as to direct alterations of the metabolism produced by the herbicide in the host plant, displayed as a root growth reduction. Similarly to the growth parameters, the inoculation with the saprophytic fungi restored the inhibition of the nodulation and the nitrogen fixation rate, which would be associated with the degradation of pendimethalin in the soil (Fig. 5). In the case of *F. lateritium*, a significant reduction in nodule biomass was observed, which could be related to the quick establishment of the fungal mycelium in the soil, that might be inducing a defensive response in the plant with a negative effect in nodule development, as previously shown in a tripartite interaction in which *F. oxysporum* reduced the nodulation in *Medicago truncatula* (Batnini et al. 2023).

Different field dissipation studies have revealed a relatively high persistence of pendimethalin in the soils, depending on soil properties and climatic circumstances (Jaźwa et al. 2009; Sondhia 2013). In this respect, pendimethalin degradation in soil may naturally occur through a combination of different processes such as photodegradation, evaporation, volatilization, leaching, and biodegradation (Kaur et al. 2025; Strandberg and Scott-Fordsmand 2004). Moreover, indigenous soil microorganisms can develop an adaptability to degrade pendimethalin following repeated application, leading to a faster dissipation of the herbicide in the surface soil (Wayment et al. 2025). All these factors may lead to a very wide range of half-life values of pendimethalin in soil (Kocárek et al. 2016). In our experimental conditions under a controlled environment, about 40% of pendimethalin dissipated after 28 days, which is in accordance with dissipation rates reported by previous studies (Kewat et al. 2001; Kocárek et al. 2016; Kulshrestha et al. 2000). However, inoculation with both saprophytic fungi significantly increased the dissipation between 80% and 90%, depending on the fungal species, which indicates the capacity of both fungi to degrade pendimethalin in soil, similarly to other fungal species (Barua et al. 1990; Bravim et al. 2021; Kulshrestha et al. 2000). These findings

encourage the use of these fungi for the bioremediation of soils containing elevated concentrations of this herbicide, as they can be inoculated to act as pollutant-degrading microorganisms in scenarios where no specific microbial degraders are present within the indigenous population (Pinto et al. 2012; Gabriela et al. 2023).

Soil enzymatic activities are essential for nutrient cycling and microbial function. In this study, pendimethalin reduced β -glucosidase activity by 30% in non-inoculated soils (Table 2), consistent with previous reports on herbicide-induced microbial disruption (Prudnikova et al. 2020). However, inoculation with *C. rigida* and *F. lateritium* mitigated this effect, highlighting their potential role in maintaining soil functionality under chemical stress (Dennis et al. 2023). Interestingly, *F. lateritium* inoculation reduced β -glucosidase (45%) and phosphatase (35%) activity in untreated soils, likely due to microbial competition (Nannipieri et al. 2018). However, phosphatase activity increased by 30% in herbicide-treated soils with *F. lateritium*, suggesting an adaptive response enhancing phosphorus availability (Baćmaga et al. 2023). Urease and basal respiration remained unaffected, indicating microbial resilience or limited herbicide interference in nitrogen cycling (Chen et al. 2021). Overall, *C. rigida* and *F. lateritium* showed potential in reducing herbicide-induced enzymatic disruption, but their effects vary, underscoring the need for targeted bioremediation strategies.

In addition to increasing the dissipation of pendimethalin, fungal inoculation caused an increment in the amounts of bacterial and fungal biomass (Fig. 6), reinforcing the role of fungal amendments in promoting microbial proliferation (Mayer Labba et al. 2021). The increment in the fungal biomass was significantly higher in the soil inoculated with *F. lateritium*, suggesting that this fungi established itself more effectively in the soil than *C. rigida*. The effect of the fungal inoculation in the soil bacterial abundance might be associated with the degradation of pendimethalin, that would be constraining the soil microbial development in non-inoculated soils (Gabriela et al. 2023). In relation to the soil microbiological status, the presence of pendimethalin did not significantly affect the overall abundance of bacteria and fungi (Fig. 6), suggesting microbial resilience to the herbicide, as previously reported for certain agroecosystems (Dennis et al. 2023).

PERMANOVA results revealed that while pendimethalin did not significantly alter microbial community structure, fungal inoculation and its interaction with the herbicide did. This aligns with findings that fungal amendments can drive shifts in microbial communities by modifying resource availability and competitive interactions. Studies have shown that pendimethalin application can reduce microbial counts in soil, particularly affecting fungal populations such as

Aspergillus, *Rhizopus*, and *Penicillium* (Oyeleke et al. 2011). However, recent research indicates that pendimethalin has minimal effects on bacterial and archaeal communities but can influence the abundance of specific operational taxonomic units (OTUs) after prolonged exposure (Dennis et al. 2023). Additionally, fungal consortia, including *Aspergillus fumigatus* and *Penicillium citrinum*, have been shown to degrade pendimethalin, indicating that fungal inoculation may play a role in altering microbial community dynamics (Bravim et al. 2020). NMDS analysis further confirmed that inoculation was the primary factor influencing community differentiation, with *F. lateritium* having a stronger impact than *C. rigida*. This suggests that *F. lateritium* may induce more profound microbial shifts, possibly due to its metabolic activity or interactions with native soil biota. Bioremediation treatments should have as little effect as possible on the native soil microbial community (Mawarda et al. 2020). Therefore, the effects of *C. rigida* and *F. lateritium* on the structure of soil bacterial and fungal communities should be evaluated using more reliable approaches, such as metabarcoding of the 16 S rRNA gene and ITS2 region, rather than relying solely on changes in the relative abundance of fatty acids, as done in this study. If the significant impact of *F. lateritium* on native soil microbial communities is confirmed, it will be necessary to identify a dosage that minimizes disruption to the microbial community while still effectively reducing pendimethalin concentrations in the soil.

Regarding the overall effect of the treatments on both soil and plant status, the PCA analysis suggests that all treatments led to contrasting responses under the experimental conditions, particularly in their influence on parameters such as nodulation, nitrogen fixation rate, soil enzymatic activities, and plant growth (Fig. 7). The differentiation between treatments with *F. lateritium* and *C. rigida* suggests that each fungus generates distinct effects on nodulation and plant growth. The presence of herbicide-treated plants within both fungal groups indicates that, despite its application modulates plant responses, possibly affecting nitrogen fixation, enzymatic activity, and biomass development, the inoculation of fungi minimises its negative influence. In this sense, the clear separation of the group representing the herbicide-treated not inoculated plants reinforces the hypothesis that herbicide application negatively impacts plant-microorganism symbiosis. This is reflected in affected nodulation, reduced nitrogen fixation efficiency, and altered soil enzymatic activity, ultimately having a direct impact on plant growth.

Furthermore, the main variables, such as nitrogen fixation rate and growth parameters, were strongly correlated with certain treatments, especially those involving fungal inoculation, regardless of the application or not of the herbicide. This suggests that plant-fungus interaction directly

influences nitrogen uptake and overall plant development diminishing the adverse effect of the herbicide application, which are manifested in the herbicide non-inoculated treatment. Moreover, soil enzymatic activities, including β -glucosidase, phosphatase, and urease, also played a key role in nutrient cycling and microbial activity in the rhizosphere. The distribution of treatments suggests that fungal inoculation, particularly in the absence of herbicide, may enhance enzymatic activity, potentially improving nutrient availability and symbiotic efficiency. Therefore, the results obtained through the PCA analysis highlight the importance of evaluating the interaction between different fungal species, soil enzymatic activities, and nutrient availability to optimize plant growth under various experimental conditions related to the presence or absence of herbicide.

5 Conclusions

In this work, we provide evidence of the adverse effects of pendimethalin application on the symbiotic responses of *P. vulgaris* by the reduction of nitrogen fixation and AMF root colonization. Additionally, we confirm the effectiveness of *C. rigida* and *F. lateritium* to be used as a bioremediation tool for the removal of pendimethalin to improve soil microbial activity and the symbiotic interactions between soil microbes and legumes to contribute to agricultural sustainability. Further experiments would be needed to test the efficacy of both fungi under field conditions.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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