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RESEARCH

Enhancing chickpea growth through arbuscular mycorrhizal fungus inoculation: facilitating nutrient uptake and shifting potential pathogenic fungal communities

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Abstract

Arbuscular mycorrhizal fungi (AMF) are the most widespread plant symbionts associated with plant roots, and theyperform numerous functions that contribute to plants' health and physiology. However, there are many knowledge gaps in how the interactions between AMF and root mycobiomes influence the performance of the host plants. To this end, we inoculated a local chickpea cultivar grown in agricultural soil under semi-controlled conditions with *Rhizophagus irregularis*. In addition to examining mycorrhizal colonization, plant biomass, and mineral nutrition, we sequenced the ITS region of the rDNA to assess the chickpea mycobiome and identify key fungal taxa potentially responding to *R. irregularis* inoculation. Our results showed that inoculation had a positive effect on chickpea biomass and mineral nutrition, especially the total aboveground phosphorus, potassium and sodium contents. *Fusarium*, *Sporomia*, *Alternaria*, and unknown Pleosporales were the most abundant taxa in the roots, while *Stachybotris*, *Penicillum*, *Fusarium*, *Ascobolus*, an unknown Pleosporales and *Acrophialophora* were the most abundant in the rhizosphere. Among the ASVs that either were enriched or depleted in the rhizosphere and roots are potential plant pathogens from the genera *Didymella*, *Fusarium*, *Neocosmospora*, and *Stagonosporopsis*. This study highlights the relevance of AMF inoculation not only for enhancing chickpea growth and mineral nutrition in semi-arid conditions but also for influencing the composition of the plants' fungal community which contributes to improved plant performance and resilience against biotic and abiotic stress.

Keywords Arbuscular mycorrhizal symbiosis · Phosphorus fertilization · Fungal community composition and structure · Pathogenic fungi · Biostimulant · Food security

Introduction

Fungal communities — also known as mycobiomes — inhabiting plant roots and rhizospheres play essential roles in plant physiology and ecology. These plant mycobiomes comprise various groups of fungi with distinct roles and functions, the most significant being beneficial and pathogenic fungi. Beneficial fungi, such as mycorrhizal fungi and endophytic fungi, can directly or indirectly enhance plant growth and induce tolerance to biotic and abiotic stress, leading to improved plant performance. Conversely, pathogenic fungi can harm crop growth, leading to yield losses and posing a major threat to agricultural crop production. Therefore, studying plant mycobiomes is essential for improved disease management, ecological practices, and the development of eco-friendly crop production methods (Anal et al. 2020). However, little is known about the diversity and functions of the mycobiomes of major food crops as well as their responses to environmental changes and agricultural practices, especially bioinoculation.

The chickpea (*Cicer arietinum* L.) is a high-value legume crop that is pivotal for achieving food security, addressing climate change issues, and managing soil degradation, because

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of its low agricultural input demand, enabling the crop to perform well on marginal soils. Two types—*Desi* (small and pigmented) and *Kabuli* (large and non-pigmented)—are consumed widely across the globe, owing to their high protein (20–30%) and carbohydrate (40%) contents, in addition to being good dietary sources of minerals, such as calcium, phosphorus, magnesium, iron, and zinc, pro-vitamins, such as beta-carotene, and fiber (Kudapa et al. 2018; Kibar et al. 2023). Thus, it is unsurprising that chickpeas rank second after soybeans among the highest-produced food legumes in the world, with an estimated 18,095,248 tons produced in 2022 (FAOSTAT 2023). The *Kabuli* chickpea is the main chickpea type cultivated in Morocco, where it constitutes the second-largest food legume crop grown (after faba beans), with an annual production rate of 75,000 tons and 30,953 tons in 2021 and 2022, respectively, according to FAOSTAT (FAOSTAT 2023).

Morocco's chickpea productivity decreased by more than half between 2021 and 2022, with the country's total production dropping to the fifth position in Africa in 2022, as compared to the fourth position held in 2021 (FAOSTAT 2023). Although chickpeas are widely cultivated in many semi-arid and arid climates, the crop faces many challenges, including climatic factors such as drought and heat, pathogen attacks, and low adoption of certified seeds (Houasli et al. 2020; Mohammed et al. 2017; Arriagada et al. 2022). Like other countries in the Middle East and North Africa region, Morocco's agriculture is severely affected by drought and excessive heat conditions, leading to diminished agricultural productivity. Long-term drought and erratic rainfall decreased Morocco's agricultural productivity by approximately 17% in 2022 (Tsakok 2023). The recent decline in chickpea productivity can be attributed to sparse rainfall and excessive heat experienced in recent years, given that chickpea cultivation in most fields is rainfed.

Fortunately, methods that improve phosphorus-use efficiency in chickpeas have been demonstrated to enhance chickpea growth under drought stress in Moroccan semi-arid pedoclimatic conditions (Chtouki et al. 2022a; Khadraji et al. 2017). Increasing P supply to legumes can stimulate nodule formation leading to N_2 fixation (Cabeza et al. 2024; Zhong et al. 2023).

In addition to abiotic stress, fungal pathogens—notably, members of the *Didymella* and *Fusarium* genera—are harmful for many chickpea cultivars grown locally (Krimi Bencheqroun et al. 2022). For example, annual yield losses because of pathogen infestations, especially *Fusarium* wilt/root rot, can reach 90–100% in many fields (Elbouazaoui et al. 2022). Thus, improving chickpeas' resilience to both abiotic and biotic stresses holds a significant promise not only for enhancing food security, but also for promoting economic prosperity in the region.

Arbuscular mycorrhizal fungi (AMF) are widely distributed plant symbionts that colonize the roots of most terrestrial plants, including agriculturally important crops (Basiru et al. 2020). AMF inoculation can assist crops in adapting to various abiotic stresses, especially nutrient limitation and water scarcity (Abdalla et al. 2023; Kavadia et al. 2020), resulting in a substantial yield increase (Hijri 2016). The benefits attributed to AMF inoculation often pertain to nutrient acquisition, which usually relies on the soil's physicochemical characteristics, especially soil phosphorus availability; however, recent evidence indicates that soil biotic communities, both micro- and macrofauna, can strongly predict the success of AMF inoculation in field conditions (Zhai et al. 2023; Zhang et al. 2019). A large-scale study across 54 fields demonstrated that the abundance of pathogenic species, rather than nutrient availability, was the most reliable predictor of plants' growth response to AMF inoculation (Lutz et al. 2023). That is, declines in several soilborne pathogens in maize roots following AMF inoculation were positively correlated with plant growth in fields where the mycorrhizal growth response was positive (Lutz et al. 2023). Hence, it may be inferred that the colonization of roots by AMF could offer advantages in enhancing plants' performance by suppressing potential plant pathogens in roots and in the rhizosphere (Ismail and Hijri 2012; Ismail et al. 2011, 2013), in addition to promoting nutrient acquisition. Nevertheless, the efficacy of this capability remains insufficiently examined across agriculturally significant ecosystems and crops.

Recently, there have been increasing efforts to improve chickpeas' performance under dry climates through non-diazotroph microbial inoculation, especially with arbuscular mycorrhizal fungi and endophytic fungi (Gomez et al. 2024; Rocha et al. 2019); however, growth responses sometimes differ widely across chickpea genotypes (Kavadia et al. 2020; Bazghaleh et al. 2015). It was demonstrated that two chickpea genotypes responded differently to water limitation because of their contrasting abilities to associate with AMF: the AMF-responsive genotype showed greater tolerance to water limitation than the other genotype (Kavadia et al. 2020). Moreover, the successful establishment of foreign AMF strains in a new area outside their local ecological range is often challenged by competition from native communities, leading to inconsistent results (Basiru and Hijri 2022a). After establishment, root colonization by AMF can shape the host crop's microbiome, reflected in plants' phenotypic expression; however, the extent of this influence is still contingent on geographic location, soil properties, and plant genotype (Li et al. 2021; Lutz et al. 2023). Regrettably, little-to-no information exists on how inoculation with non-native AMF can impact chickpeas' mycobiome, and consequently affect the host plants' health and physiology.

Therefore, the objective of this study was to investigate whether chickpeas inoculated with a non-native arbuscular mycorrhizal fungus can perform better under a semi-arid climate than non-inoculated chickpeas that are colonized only by indigenous AMF. We selected *Rhizophagus irregularis* DAOM 197,198, originally isolated from Pont-Rouge, Quebec, Canada, as it is the most commonly used AMF isolate in commercial inoculants (Basiru et al. 2020). Our hypotheses were as follows: (1) inoculation with *R. irregularis* enhances chickpeas' growth and augments its mineral nutrition under low-phosphorus conditions in calcareous soil; (2) inoculation with *R. irregularis* affects the community composition of the rhizosphere and root mycobiome of chickpeas; and (3) inoculation with *R. irregularis* stimulates or suppresses certain key fungal taxa that contribute to the health of chickpeas, thus acting as indicators of the mycorrhizal fungus inoculation's success in both chickpea roots and the rhizosphere habitats. To test these hypotheses, we conducted a pot experiment in a naturally lit greenhouse using locally bred *Kabuli* chickpea plants (cultivar Moubarak) inoculated with *R. irregularis* DAOM 197,198, a non-native AMF strain. We assessed mycorrhizal colonization, plant biomass, and mineral nutrition. Additionally, we utilized ITS metabarcoding to examine changes in the chickpea mycobiome, and we conducted differential abundance and regression analyses to identify key taxa in the mycobiome that may influence the chickpeas' growth response to inoculation.

Materials and methods

Setup of the semi-controlled experiment

The experiment was conducted at UM6P's experimental farm in Ben Guerir, Morocco. The average daily temperature was 22/15°C (day/night), and the average relative humidity was 65%. The soil used in the study was collected from the experimental farm prior to mixing with river sand at a ratio of 2:1 (v: v). The mixed soil and sand were sieved through a 4 mm mesh before being used to fill 10 L pots. Composite samples of this soil mixture were taken randomly for chemical analysis (Table S1). The AMF strain *Rhizophagus irregularis* DAOM197198 (originally isolated from Pont Rouge, Quebec, Canada) was used for the inoculation; it was propagated in vitro in Medium M using DNA-transformed chicory roots for 4 months.

Chickpea seeds (cultivar Moubarak) were planted on March 08, 2021, at a rate of five seeds per pot, which was reduced to three seedlings after germination. The study was part of a larger experiment aimed at investigating the response of chickpea growth and physiology to three biostimulants, including *R. irregularis*. Each biostimulant was treated as an independent factor with two levels, with *R. irregularis* either included or excluded in each treatment. The experiment was replicated seven times, with *R. irregularis* present or absent. In total, there were seven blocks; four blocks were harvested in the first round (Harvest 1), while three blocks were left to mature and were harvested in the second round (Harvest 2). Overall, 32 pots from the first harvest and 24 pots from the second harvest were included in the analyses for this study.

The inoculum consists of spores, hyphae, and carrot roots that were utilized for propagation. To prevent post-irrigation leaching of the inoculum, a piece of filter paper was placed beneath each seed as shown in Fig. S1. To enhance early establishment of the applied mycorrhizal fungus, the inoculum was applied as a seed treatment in order to initiate a priority advantage (Basiru and Hijri 2022b). To eliminate discrepancies between inoculated and non-inoculated treatments, filter papers was applied to all seeds. No mock inoculation was performed; instead, the non-inoculated control received water equivalent to the volume of the inoculum applied.

Fertigation (the application of mineral nutrients through irrigation) was carried out twice at a four-week interval, with total nutrient contents of N (25 kg/ha), P (30 kg/ha), and K (25 kg/h) as ammonium phosphate, potassium nitrate, and ammonium nitrate (Table S2). The systemic fungicide Thiophanate Methyl was applied as a foliar spray at a dose of 100 g/hectoliter six weeks after sowing, following the observation of lesions suspected to be Anthracnose on the shoots of the chickpea plants.

Experimental harvest and measurement of biomass and yield parameters

Harvest 1 To assess the biomass and chickpea root mycobiome, four blocks were randomly selected for harvest on May 18, 2021, corresponding to 70 days after seeding (approximately 60 days after seedling emergence). Each pot, containing three chickpea plants, was harvested destructively. Rhizosphere soil (soil closely associated with chickpea roots) was collected using sterilized scapulae. After harvesting the shoots by cutting the plants just above the soil line, the roots were gently removed from the pots and washed under running tap water. Fine root samples were collected and stored together with the rhizosphere soil in a cooling box before being transported to the laboratory, where they were stored at -20 °C for subsequent analyses.

Harvest 2 The remaining three blocks were harvested on June 30, 2021 (113 days after seeding), after reaching full maturity, to determine biomass yield and root trait

parameters. The shoot and root samples were harvested using the same method as in Harvest 1. Fresh weights were recorded, and the samples were then dried in an oven at 70 °C for 48 h to determine dry biomass. Surprisingly, we did not observe any nodules on the roots at either harvest.

Root imaging and mycorrhizal colonization

The root colonization was examined at Harvest 1. The method was based on the ink–vinegar staining technique, which is considered to be safer and more easily executable than other staining techniques (Vierheilig et al. 1998). Briefly, the roots were cut into small fragments of 1–2 cm and cleared with 10% KOH in a microwave at full power for 2 min. The cleared roots were subsequently rinsed with acidified tap water and stained with 5% ink in vinegar. Following staining, 20 root fragments were positioned atop a slide and observed under an optical microscope to examine both the number of colonized roots and the intensity of colonization (Trouvelot 1986). Furthermore, root samples were scanned to produce images, which then were analyzed using WinRHIZO version 2003b (Regent Instruments Inc., Quebec, Canada) to measure root length, average diameter, and volume.

Mineral content analyses

To determine the shoots' mineral contents, shoot biomass was harvested at Harvest 1 and dried at 70 °C for 72 h to constant weight. Samples including flowers and emerging pods were ground into a fine powder and sent for chemical (N, P, K, Na, Zn) analysis by the soil analysis laboratory of the Agricultural Innovation and Technology Transfer Center (AITTC), at the University Mohammed VI Polytechnic (UM6P).

DNA extraction

Approximately 250 mg soil samples obtained from the rhizosphere were used for each treatment, while 50 mg of the lyophilized roots were ground into a powder using tungsten beads in a TissueLyser II (Qiagen, Global Diagnostic Distribution, Temara, Morocco). DNA extraction was carried out using both DNeasy PowerSoil and DNeasy Plant kits according to the manufacturer's instructions. Subsequently, DNA was quantified using a Qubit Fluorometer (Thermo Fisher, Rabat, Morocco). Extracted DNA samples were stored at -20 °C until use.

PCR amplification and library prep

The fungal ITS2 region was amplified using the following primer pair containing adapters ***CS1***_ITS3_KYO2 (5'-ACACTGACGACATGGTTCTACAGATGAAGAACGYAGYRAA-3') and ***CS2***_ITS4 (5'-TACGGTAGCAGAGACTTGGTCTTCCTCCGCTTATTGATATGC-3'). The PCR reaction was performed in a 25 µL reaction volume consisting of 1X Platinum Direct PCR Universal Master Mix (Thermo Fisher, Rabat, Morocco), 0.25 µM of each primer, 1X Platinum GC Enhancer, and approximately 20 ng of template DNA. Thermal cycling was carried out using an Eppendorf Mastercycler Pro PCR instrument (Eppendorf, Hamburg, Germany) with the following cycling conditions: an initial denaturation step of 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min, with a final elongation step of 10 min at 72 °C. Each PCR run included negative PCR controls containing only water, and no visible amplification was observed. Amplified products were quantified and visualized on a 1% agarose gel. Subsequently, amplicons were submitted for library preparation and sequencing using Illumina MiSeq PE 2×300 bp (Legeay et al. 2024b). The sequences were demultiplexed by the service provider.

Bioinformatics

The DADA2 pipeline implemented in R version 4.3.2 was utilized to analyze raw reads (Callahan et al. 2016). Reads exceeding the expected error rate by more than two were discarded, and forward and reverse reads were trimmed at 50 and 100 bp, respectively, due to the low quality at those termini. Chimera sequences were excluded utilizing the consensus method. The resulting amplicon sequence variants (ASVs) underwent taxonomic assignment using the UNITE database (Nilsson et al. 2019) and the default parameters of the "assignTaxonomy" command.

ASVs, taxonomy table and metadata files were imported into phyloseq (version 1.46.0) (McMurdie and Holmes 2013). The reads corresponding to plant DNA, amounting to 30% of reads in the root samples, were discarded. The raw data were transformed into compositional data using the phyloseq package (McMurdie and Holmes 2013).

Alpha diversity indices (Shannon, Simpson and Chao1) were computed using the Vegan package with the *estimate_richness* function (Ssekagiri et al. 2017), visualized using ggplot2 package (Wickham 2016). The effects of biotopes (roots and rhizosphere) and inoculation on each index were assessed using the Kruskal-Wallis test, utilizing the stat_compare_means function from the ggpubr package (Kassambara 2020). Beta diversity analysis was conducted based on Bray-Curtis ecological distances using the compositionally

transformed ASV counts. Subsequently, global dissimilarity between subjects was evaluated through principal coordinate analysis (PCoA) and visualized using the ordinate and plot_ordination functions from the microbiome package. To test the effects of biotopes and *R. irregularis* inoculation on ecological distance, permutational multivariate analysis of variance (PERMANOVA) was performed using adonis2 from the Vegan package (Oksanen et al. 2015).

Differential abundance analysis was carried out using the microbiomeSeq (version 0.1) package (Ssekagiri et al. 2017). Briefly, this package applies the DESeq2 procedure, including parametric fitting, the Wald test, and a significance threshold of 0.05 adjusted by the family-wise error rate (FWER) (Love et al. 2014). Two separate analyses were conducted on root and rhizosphere soil samples, and the log2 fold change of significantly enriched or depleted ASVs was plotted using ggplot2 (Wickham 2016).

Statistical analysis

To estimate the effect of inoculation on chickpea biomass, mineral nutrition, and pod yield, a linear mixed model was fitted using the *lmer* function from the lme4 package (version 1.1–35.1) (Bates et al. 2015). *R. irregularis* inoculation (together with other biostimulants of the entire original experiment) were considered fixed variables, while block was considered a random factor. Only the independent effects of *R. irregularis* inoculation are reported. Prior to model fitting, data from the pot experiment were examined for normality and homogeneity of variance and the residual distributions of the model were visually inspected through QQ plots. The model was then subjected to analyses of variance using the *anova* function from the LmerTest package (version 3.1-3) (Kuznetsova et al. 2017) based on Satterthwaite's method. To determine the difference between inoculated and non-inoculated treatments, the Tukey pairwise comparison test was computed by the *emmeans* function from the emmeans package (Lenth and Lenth 2018). All bioinformatics and statistical analyses were conducted in R (version 4.3.2) (R Core Team 2021) using R Studio software.

Results

Effects of treatments on root colonization, biomass and mineral nutrition of chickpea

Microscopic observation showed the presence of AMF in chickpea roots regardless of inoculation status (Fig. S2). Inoculation did not significantly affect the frequency or intensity of AMF colonization in chickpea roots ($p = 0.62$) (Fig. 1).

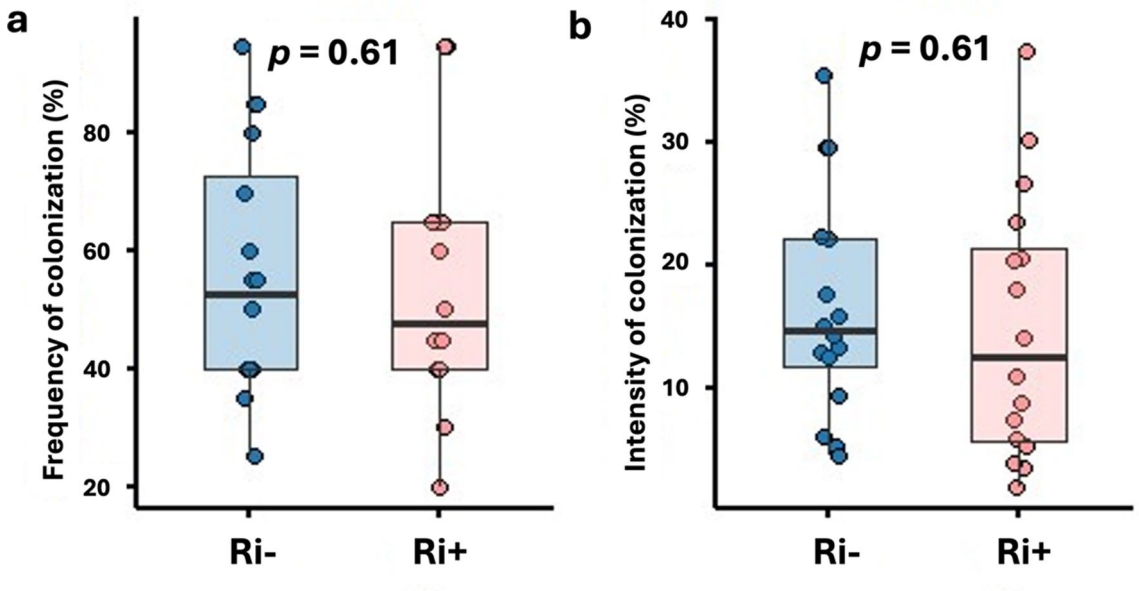

Fig. 1 Mycorrhizal colonization of chickpea roots following inoculation with *R. irregularis*. The boxplots show (**a**) the frequency and intensity of mycorrhizal colonization in chickpea roots at Harvest 1. Treatments without and with *R. irregularis* inoculation are denoted as Ri- and Ri+, respectively. Boxes represent the 1st (Q1) and 3rd (Q3) quartiles, with the midline showing the median. The upper whisker extends from the 75th percentile (Q3) to the highest data point within 1.5 times the IQR above Q3, while the lower whisker extends from the 25th percentile (Q1) to the lowest data point within 1.5 times the IQR below Q1. Data points outside the whiskers are considered outliers while each point represents a single replicate of three chickpea plants per pot. Sample size (n) is 4, and p-values are considered significant at < 0.05

Despite the lack of a significant difference in mycorrhizal root colonization between control and inoculated chickpea plants, *R. irregularis* inoculation enhanced shoot biomass at Harvest 1 (70 DAS) (9%, $p = 0.01$) but tended to decrease root biomass (-7.6%, $p = 0.23$). A similar trend was observed at Harvest 2 (113 DAS), in which inoculated chickpea plants had higher shoot biomass (which increased by 14%, $p = 0.04$) than controls, however, the effect of inoculation on root biomass, pod dry weight and total aboveground biomass were not significantly different from that of controls (Fig. 2).

Moreover, *R. irregularis* inoculation positively influenced nutrient uptake in chickpea by significantly promoting aboveground P (12%, $p = 0.03$), K (9.5%, $p = 0.02$) uptake (Fig. 3). Inoculation also marginally increased Na (11%, $p = 0.05$) concentration, but had no significant effect on N ($p = 0.67$; Fig. 3) or Zn ($p = 0.63$) concentration (data not shown).

Fungal raw sequences and composition

A total of 973 ASVs were identified, encompassing seven phyla, 27 classes, 60 orders, 137 families, 244 genera, and 350 species. Ascomycetes dominated the fungal communities of both the rhizosphere soil and root samples, accounting for more than 90% of all phyla, followed by Basidiomycetes, Chytridiomycetes, Glomeromycetes, Monoblepharomycetes, Mucoromycetes, and Rozellomycetes (Fig. S3). Sodarimycetes, Dothideomycetes, and Glomeromycetes constituted the major taxa in the roots, while Sordariomycetes, Dothideomycetes, Eurotiomycetes, Pezizomycetes, constituted the major taxa in the soil samples at the class level (Fig. 4a). At the family level, Nectriaceae, Pleosporaceae, an unknown Pleosporales family, Didymellaceae, and Stachybotryaceae had the greatest abundance in chickpea roots, whereas in the soil compartment, the fungal families were more diverse, including major taxa such as Nectriaceae, Stachybotryaceae, Sporomiaceae, Aspergillaceae, Chaetomiaceae, Didymellaceae, Ascobolaceae, Pleosporaceae, and

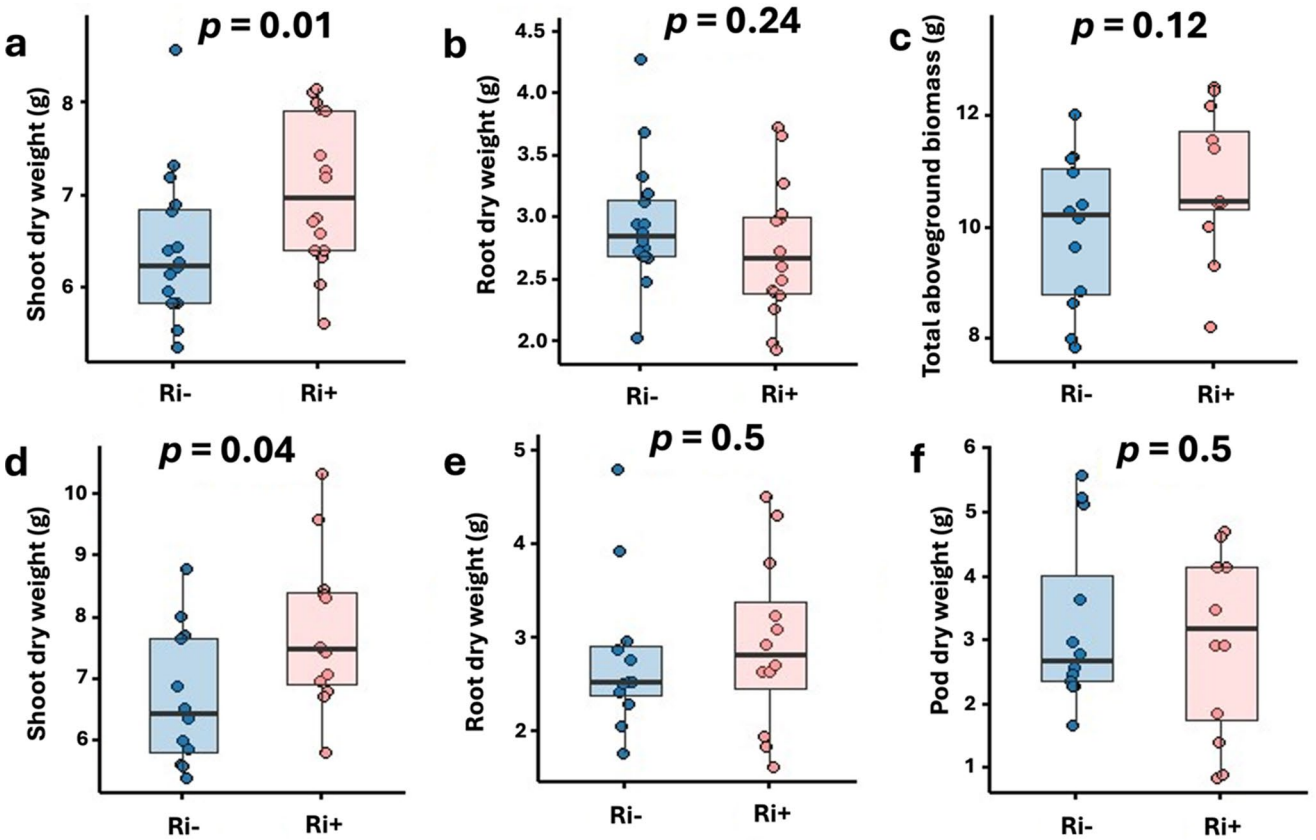

Fig. 2 Chickpea biomass and yield under *R. irregularis* inoculation. The boxplots display (**a**) shoot biomass and (**b**) root biomass at Harvest 1, as well as (**c**) total aboveground biomass, (**d**) shoot biomass, (**e**) root biomass, and (**f**) pod yield at Harvest 2. Treatments without and with *R. irregularis* inoculation are denoted as Ri- and Ri+, respectively. Boxes represent the 1st (Q1) and 3rd (Q3) quartiles, with the midline showing the median. The upper whisker extends from the 75th percentile (Q3) to the highest data point within 1.5 times the IQR above Q3, while the lower whisker extends from the 25th percentile (Q1) to the lowest data point within 1.5 times the IQR below Q1. Data points outside the whiskers are considered outliers and each point represents a single replicate of three chickpea plants per pot. Sample sizes are $n = 4$ for Harvest 1 and $n = 3$ for Harvest 2. *P*-values are considered significant at < 0.05

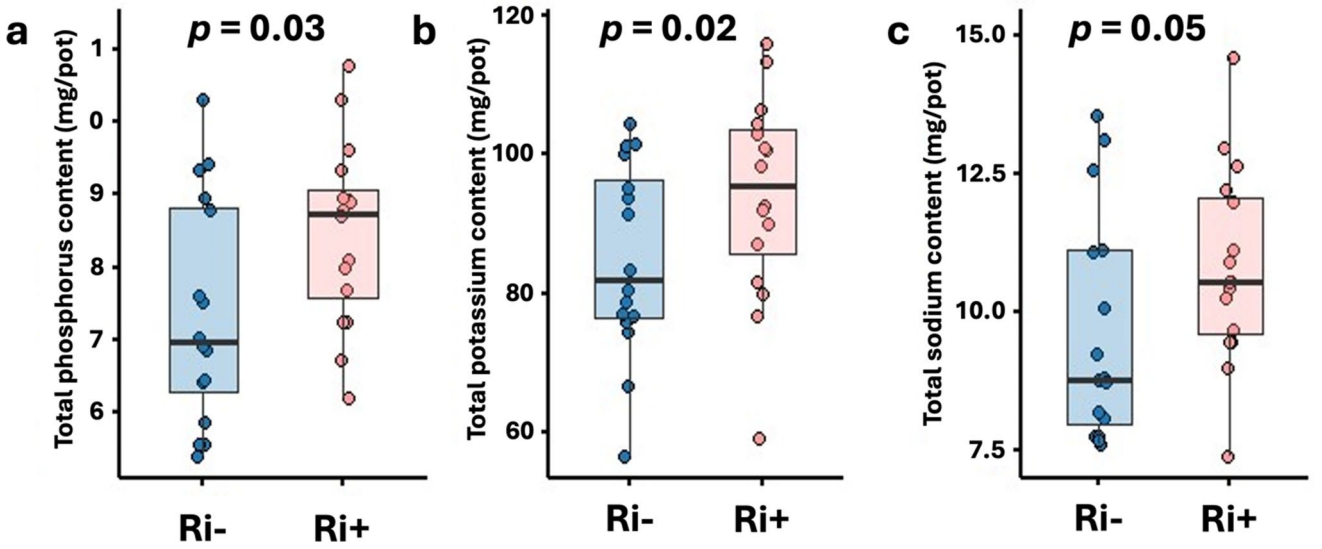

Fig. 3 Chickpea nutrient uptake in response to *R. irregularis* inoculation. The boxplots illustrate (**a**) phosphorus, (**b**) potassium, and (**c**) sodium contents at harvest 1. Treatments without and with *R. irregularis* inoculation are denoted as Ri- and Ri+, respectively. Boxes represent the 1st (Q1) and 3rd (Q3) quartiles, with the midline showing the median. The upper whisker extends from the 75th percentile (Q3) to the highest data point within 1.5 times the IQR above Q3, while the lower whisker extends from the 25th percentile (Q1) to the lowest data point within 1.5 times the IQR below Q1. Data points outside the whisker ranges are considered outliers each point represents a single replicate of three chickpea plants per pot. Sample size is $n=4$, and p-values are considered significant at <0.05. Sample size (n)$=4$, and p-values are considered significant at <0.05

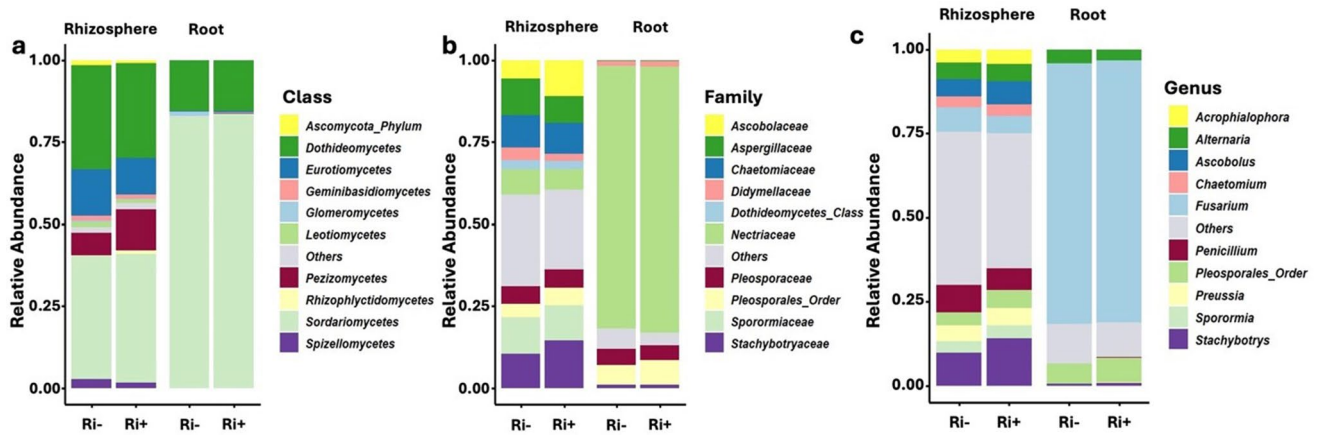

Fig. 4 Composition of chickpea fungal communities in the rhizosphere and root biotopes. Relative abundance of fungi at the (**a**) class, (**b**) family, and (**c**) genus levels following *R. irregularis* inoculation. Ri+/Ri- indicates treatments with or without *R. irregularis* inoculation

an unknown Pleosporales family (Fig. 4b). Among the most prevalent genera, *Fusarium*, *Sporomia*, *Alternaria*, and an unidentified genus of *Pleosporales* are the most abundant in the roots, while *Stachybotris*, *Penicillium*, *Fusarium*, *Ascobolus*, unidentified *Pleosporales*, and *Acrophialophora* dominate in the rhizosphere (Fig. 4c).

Effects of inoculation on fungal alpha and beta diversities

The alpha diversity indices of the fungal communities showed significant differences between inoculated and non-inoculated controls. *R. irregularis*-inoculated samples exhibited notably lower Shannon, Simpson, and Chao 1 indices in the rhizosphere soil ($p=0.01$, 0.004, and 0.04, respectively). However, the effect on alpha diversity in the roots was not significant, although there appeared to be a reduction in Shannon and Simpson indices, as indicated by a lower median in the inoculated samples compared to controls. Additionally, the alpha diversity of the rhizosphere fungal community was substantially higher than that of the roots (Fig. 5). Principal coordinate analysis indicated distinct clustering of fungal communities between the chickpea root mycobiome and rhizosphere samples, although not by

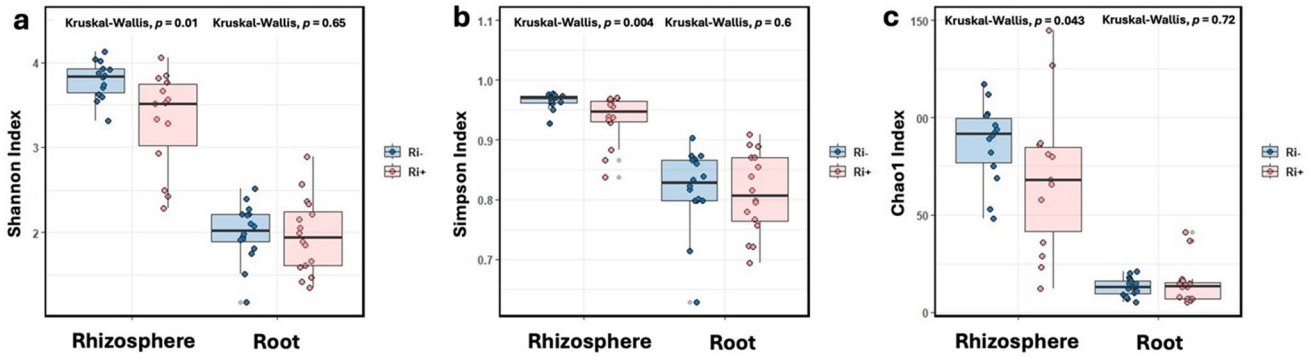

Fig. 5 Alpha diversity of fungal communities at Harvest 1 in the rhizosphere and roots of chickpea inoculated with *R. irregularis* (**a**) Shannon index, (**b**) Simpson index, and (**c**) Chao1 index for fungal diversity. Ri+/Ri- indicates treatments with or without *R. irregularis* inoculation. Boxes represent the 1st (Q1) and 3rd (Q3) quartiles, with the midline showing the median. The upper whisker extends from the 75th percentile (Q3) to the highest data point within 1.5 times the IQR above Q3, while the lower whisker extends from the 25th percentile (Q1) to the lowest data point within 1.5 times the IQR below Q1. Data points outside the whisker ranges are considered outliers. Sample size (n) = 4, and p-values are considered significant at < 0.05

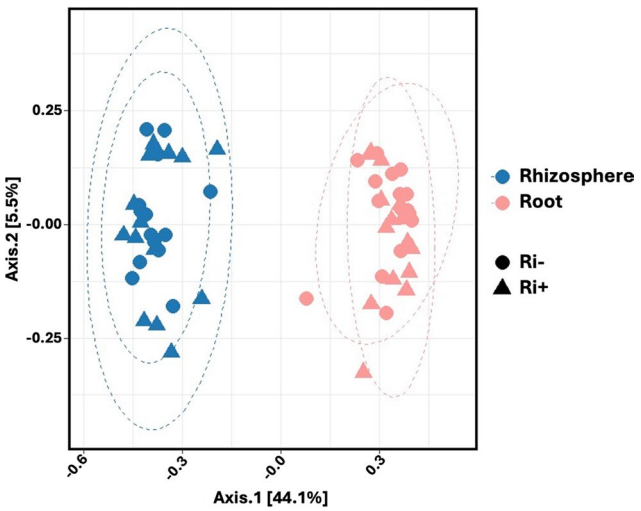

Fig. 6 PCoA plot illustrating fungal communities in chickpea roots and the rhizosphere Blue indicates rhizosphere communities, while pink represents root communities. Circular and triangular shapes correspond to Ri + and Ri- treatments, respectively, with Ri + indicating *R. irregularis* inoculation and Ri- indicating no inoculation. PERMANOVA results: Rhizosphere vs. Root, $p = 0.001$; Ri- vs. Ri+, $p = 0.3$

inoculation (Fig. 6). A subsequent PERMANOVA test confirmed a significant difference in beta diversity between root and rhizosphere communities across biotopes ($p = 0.01$), with no significant effect of *R. irregularis* inoculation on beta diversity ($p = 0.36$).

Differentially abundant taxa

To elucidate the shift in fungal community composition between inoculated and non-inoculated conditions, we conducted a differential abundance analysis on both rhizosphere soil and root biotopes. The number of ASVs exhibiting differential enrichment or depletion (log2 fold-change) was greater in soil than in roots, as presented in Table S3. Specifically, forty-three ASVs in the rhizosphere and ten in roots showed differential abundance. *Didymella arachidicola* (ASV 266), *Fusarium equiseti* (ASV 139), *Neocosmospora rubicola* (ASV 102), an unidentified Phaeosphaeriaceae (ASV 85), and an unidentified *Scutellinia* (ASV 274) were significantly enriched in *R. irregularis*-inoculated roots compared to non-inoculated roots, while *Fusarium nygamai* (ASV 109), *Neocosmospora rubicola* (ASV 102), an unidentified *Fusarium* (ASV 139), *Stagonosporopsis* (ASV 15), and *Sarocladium kiliense* (ASV 314) were depleted under *R. irregularis* inoculation (Table 1). Among the top 20 differentially abundant taxa in the rhizosphere, only six including *Rhizophlyctis rosea*, *Spizellomyces dolichospermus*, *Subramaniula*, *Acremonium persicinum*, *Fusarium nirenbergiae*, *and Penicillium* sp. were enriched. Among the depleted taxa, an unknown Spizellomycetales, *Penicillium pimiteouiense*, *Enterocarpus grenotii*, *Aspergillus bombycis*, *Scytalidium sphaerosporum*, and *Spizellomyces dolichospermus* are the most significant (Table S3 and Fig. 7).

Correlation between root fungal communities and chickpea phenotypes

Spearman correlation analyses were carried out to examine the relationship between chickpea growth phenotypes and fungal communities in roots. The results demonstrated both positive and negative relationships between root fungal communities and shoot biomass. Seven ASVs showed positive correlations with shoot biomass, including an unidentified *Stagonosporopsis* (ASV 15) ($r = 0.43$, $p = 0.01$), *Paraphoma radicina* (ASV 324) ($r = 0.42$, $p = 0.01$), *Alternaria subcucurbitae* (ASV 14) ($r = 0.40$, $p = 0.02$), *Fusarium equiseti* (ASV 29) ($r = 0.38$, $p = 0.03$), *Didymella arachidicola* (ASV 266) ($r = 0.38$, $p = 0.03$), an unidentified

Table 1 List of ASVs, their corresponding taxonomic classifications, and significance levels for differentially abundant taxa in chickpea roots

ASVs	Class	Order	Family	Genus	Species	Effect	*p*-value
ASV139	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium* sp.	Depleted	0.0036
ASV314	Sordariomycetes	Hypocreales	Hypocreales_fam_*Incertae_sedis*	*Sarocladium*	*Sarocladium kiliense*	Depleted	0.0029
ASV109	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium nygamai*	Depleted	0.00079
ASV102	Sordariomycetes	Hypocreales	Nectriaceae	*Neocosmospora*	*Neocosmospora rubicola*	Depleted	1.36E-06
ASV15	Dothideomycetes	Pleosporales	Didymellaceae	*Stagonosporopsis*	*Stagonosporopsis* sp.	Depleted	0.0029
ASV266	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*	*Didymella arachidicola*	Enriched	0.0225
ASV29	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium equiseti*	Enriched	0.022
ASV85	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Phaeosphaeriaceae genus*	Phaeosphaeriaceae species	Enriched	0.0036
ASV44	Sordariomycetes	Hypocreales	Nectriaceae	*Neocosmospora*	*Neocosmospora rubicola*	Enriched	1.36E-05
ASV274	Pezizomycetes	Pezizales	Pyronemataceae	*Scutellinia*	*Scutellinia* sp.	Enriched	0.0029

Five taxa, including an unidentified *Fusarium* species, *Sacroladium kiliense*, *Fusarium Nygamai*, *Neocosmospora rubicola*, and an unidentified *Stagonosporopsis* species, were significantly depleted in inoculated chickpea roots. In contrast, *Didymella Arachidicola*, *Fusarium equiseti*, an unidentified *Phaeosphaeriaceae* Genus, a different *Neocosmospora rubicola* ASV, and an unidentified *Scutellinia* species were enriched. Enrichment and depletion designations are based on a log2 fold change with $p < 0.05$

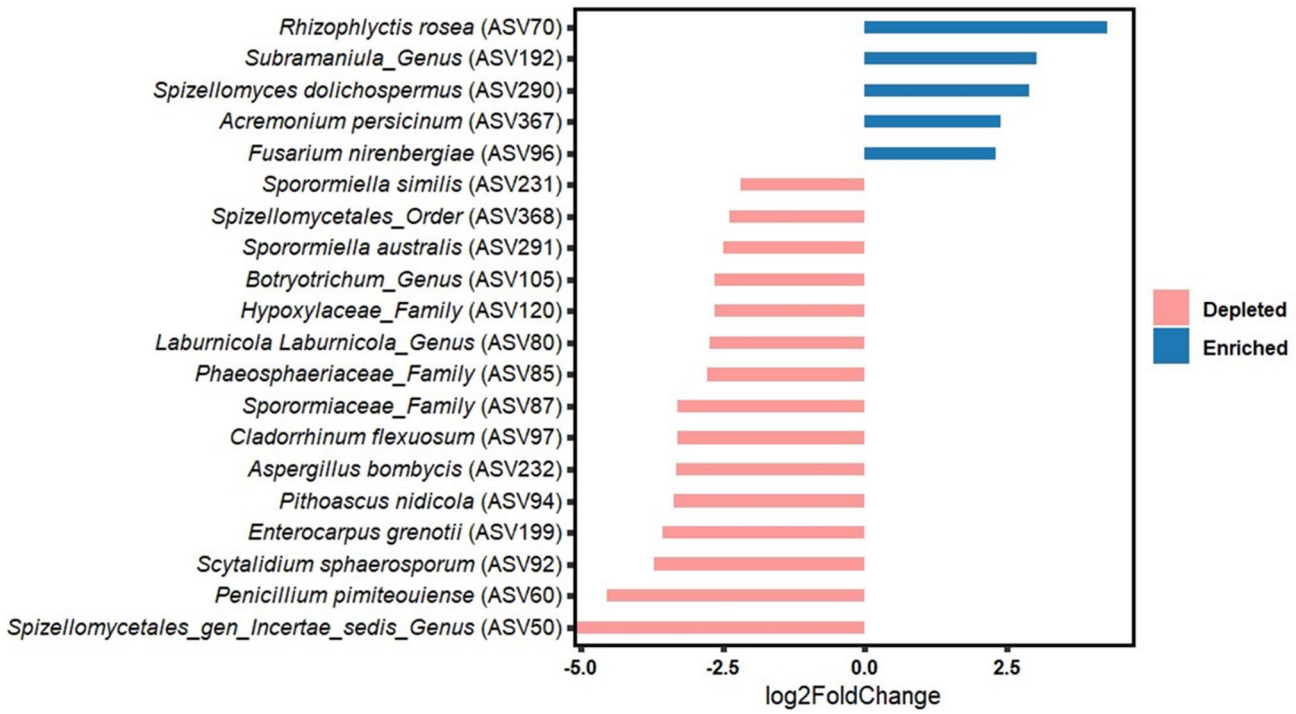

Fig. 7 Differentially abundant taxa in chickpea rhizosphere inoculated with *Rhizophagus irregularis.* Only the top 20 differentially abundant taxa are displayed. The blue bars represent enriched taxa, while the pink bars indicate depleted taxa following *R. irregularis* inoculation. All family-wise error rate-adjusted *p*-values are below 0.05

Cladosporium (ASV 77) ($r = 0.38$, $p = 0.03$), *Neocosmospora rubicola* (ASV 44) ($r = 0.37$, $p = 0.03$), and *Alternaria chlamydosporigena* (ASV 18) ($r = 0.338$, $p = 0.05$). Notably, several of these ASVs responded to *R. irregularis* inoculation, such as *Fusarium equiseti* (ASV 29), *Neocosmospora rubicola* (ASV 44), and *Didymella arachidicola* (ASV 266). Additionally, ASV 85, an unclassified species within the Phaeosphaeriaceae family, was enriched by *R. irregularis* inoculation and showed a positive although not significant correlation with shoot biomass ($r = 0.30$, $p = 0.08$). In

contrast, ASV 15 (*Stagonosporopsis*), which was depleted under *R. irregularis* inoculation, also correlated positively with shoot biomass ($r = 0.43$, $p = 0.013$). An unidentified *Fusarium* species (ASV 139) showed a negative correlation with shoot biomass ($r = -0.337$, $p = 0.05$).

Discussion

In this study, we investigated the capability of non-native arbuscular mycorrhizal fungus *Rhizophagus irregularis* inoculation to enhance the growth and mineral nutrition of chickpeas cultivated under semi-controlled conditions in a semi-arid climate. Additionally, we examined its effects on both the root and rhizosphere soil fungal communities. Through this experiment, we showed that *R. irregularis* inoculation enhanced chickpea biomass and nutrient uptake. Our study also revealed a shift in the alpha diversity of the chickpea mycobiome following inoculation, identifying specific fungal taxa that responded to *R. irregularis* inoculation.

Our findings indicate that *R. irregularis* inoculation had a significantly positive effect on chickpea shoot biomass compared to root and pod yield. Specifically, *R. irregularis* inoculation resulted in a 16% increase in shoot biomass relative to non-inoculated controls and promoted the uptake of P, K, and Na by chickpea plants. This supports our first hypothesis, which states that inoculation with *R. irregularis* enhances chickpea growth and augments its mineral nutrition under low-phosphorus conditions in calcareous soil. The significance of *R. irregularis* inoculation in promoting chickpea growth has been established previously, however, the extent of this response often depends on the genotype (Bazghaleh et al. 2018).

The growth-promoting role of AMF can primarily be attributed to their ability to facilitate nutrient uptake through the scavenging properties of their extraradical hyphae and by altering root fungal communities. It has been documented that up to 90% of a plant's P can be acquired through the arbuscular mycorrhizal symbiotic route (Ahammed and Hajiboland 2024; van der Heijden et al. 2015). Moreover, P often is the most limiting mineral nutrient in legume production; when available, it can enhance nodulation and nitrogen fixation (Chtouki et al. 2022b; Nasr Esfahani et al. 2017). Interestingly, our study revealed a positive effect of inoculation on chickpea nutrient concentrations, with AMF inoculation significantly increasing P, Na, and K concentrations compared to the controls. Consequently, it can be inferred that *R. irregularis* inoculation stimulated P mobilization from the soil, resulting in higher shoot P concentration compared to the non-inoculated control. In soils with a pH around 8.0, P tends to precipitate with calcium, which may necessitate elevated phosphorus levels for optimal plant growth (Ducousso-Detrez et al. 2022). Thus, AMF inoculation holds promise for promoting P use efficiency in pulse crops, especially in calcareous soils with high P-fixing capacity, which are the most common soil type in arid and semi-arid regions (Amrani et al. 1999).

The observed increase in shoot biomass in our study suggests that a single seed treatment with inoculant may be adequate to facilitate root colonization and promote plant growth. Previously, Rocha et al. (2019), also found that coating seeds with AMF inoculants can enhance chickpeas' shoot biomass and pod weight under both greenhouse and field conditions. It is believed that seed treatment can provide priority advantage to introduced inoculants, allowing for the quicker establishment of the inoculated strain in roots compared to native species (Basiru and Hijri 2022b; Werner and Kiers 2015). This perhaps explains the preference for seed treatment as a method of AMF application for many commercial inoculants (Basiru et al. 2021). Nevertheless, the root colonization assay conducted in this study did not demonstrate any clear priority advantage between the introduced mycorrhizal fungus and indigenous species. There was an absence of significant differences in the mycorrhizal colonization frequency and intensity between the inoculated and non-inoculated treatments at Harvest 1 after planting. Unfortunately, this study did not assess the frequency and intensity of colonization at Harvest 2. However, the sustained higher shoot biomass observed in inoculated plants compared to the non-inoculated controls at Harvest 2 suggests that inoculation via seed treatment may benefit plant growth during both the early and later stages of plant development (Fig. 2).

Traditional root staining, while effective in confirming mycorrhizal colonization, does not distinguish between indigenous and introduced AMF. In contrast, ITS metabarcoding identified specific AMF taxa present in the rhizosphere and roots of both inoculated and non-inoculated chickpeas including *Funneliformis mosseae* and *Claroideoglomus drummondii* which emerged as the most dominant species in chickpea roots, whereas *R. irregularis* was found in low abundance in both the rhizosphere and roots. However, the number of Glomeromycotan sequences was generally too low in this study to warrant any meaningful conclusion about the response of the AMF community to inoculation (Fig. S4). The limited presence of *R. irregularis* and AMF sequences in general may be because of the non-specificity of the targeted ITS region for AMF community profiling, which resulted in a low diversity of AMF in the samples. However, this does not necessarily imply a low overall abundance of Glomeromycotan sequences in the samples (Hijri et al. 2006). Previous studies have indicated that ITS is not the ideal sequence barcode for profiling AMF

communities (Iffis et al. 2017). Extended-duration studies and sampling at multiple timepoints, in conjunction with the utilization of additional AMF-specific primers targeting the small (18S) or large (28S) subunits of the rRNA gene, as well as species-specific quantitative qPCR (Badri et al. 2016), would be necessary to further unravel the nature of this interaction.

In addition to improving the nutrition and nutrient-use efficiency of chickpeas, *R. irregularis* inoculation also influenced the rhizosphere and root mycobiomes. Thus, the growth benefits observed in this study under AMF treatment can be attributed in part to the modulation of pathogenic communities in both chickpea roots and rhizosphere soil. For example, the rhizosphere of inoculated plants showed a significant reduction in alpha diversity (Fig. 5), and this decline in alpha diversity coincided with the decline of several ASVs in revealing that out of 44 ASVs responsive to *R. irregularis* inoculation, only five taxa were enriched, while the remaining were depleted in the inoculated samples compared to the controls (Supplementary Table 3).

AMF inoculation has long been recognized as a potential biocontrol tool (Ismail and Hijri 2012; Ismail et al. 2011, 2013). Three mechanisms have been hypothesized to underlie AMF's biocontrol ability: modulation of the root and soil microbial community composition, antagonism against fungal pothogens, and stimulation of plant defenses (Ismail et al. 2011). Interaction of AMF with the rhizosphere and root microbial communities can be either stimulatory or inhibitory resulting in the modulation of community composition. It has been documented that exudates secreted by AMF extraradical hyphae inhibited the conidia of *Fusarium oxysporum* f. sp. *chrysanthemi* while promoting those of *Trichoderma harzianum* (Filion et al. 1999). In this study, the abundance of several fungal taxa was reduced in the rhizosphere of chickpea including aflatoxin producing fungi such as *Aspergillus bombycis* (Peterson et al. 2001) and *Botryotrichum* sp. (Rajachan et al. 2017). Additionally, five of the ten ASVs responsive to *R. irregularis* inoculation in the roots were identified as either potential pathogens or beneficial endophytes. Among these, three *Fusarium* species—*Fusarium equiseti* (which was enriched), *Fusarium nygamai*, and an unidentified *Fusarium* (both of which were depleted)—are of significant economic importance to agriculture (Coleman 2016; Ekwomadu and Mwanza 2023). Similarly, members of Didymellaceae, including *Didymella arachidicola* (enriched) and an unidentified *Stagonosporopsis* species, have been described as pathogens of plants including chickpea (Singh et al. 2022; Addisu et al. 2023).

Among the ASVs depleted in chickpea roots, *Fusarium* sp. (Blast search identified the species as *Fusarium solani*) exhibited the highest negative correlation with shoot biomass, compared to *F. nygamai* (ASV 109), *N. rubicola* (ASV 102), and *S. kiliense* (ASV 314). *Fusarium* wilt disease caused by *F. solani* is a devastating disease of chickpeas in major areas of chickpea production (Younesi et al. 2021). This disease can lead to loss of up to 100% in highly infested fields, despite favorable growth conditions (Jendoubi et al. 2017). Several isolates of *F. solani* species, including those from Morocco, have been isolated from diseased chickpeas and were able to induce disease symptoms and reduce agronomic parameters. El Hazzat et al. (2019) demonstrated that the cultivar Moubarak (which was used in the study) was the most susceptible to *F. solani* (FS2 isolate) among other locally bred chickpea cultivars. Our results thus show that inoculating a susceptible chickpea variety (e.g., Moubarak) with *R. irregularis* potentially can confer induced resistance against pathogens.

Antagonistic interaction between AMF and *Fusarium* spp. has been documented in previous studies. For instance, the inoculation of potato with *R. irregularis* (previously named *Glomus intradices*) inhibited the growth of *Fusarium sambucinum* and also reduced the expression of trichothecene mycotoxin genes that were responsible for its virulence (Ismail et al. 2011, 2013). Moreover, inoculation of papaya with an AMF complex (MTZ01) comprising four fungi (*R. intraradices*, *F. mosseae*, *Claroideoglomus etunicatum*, and *Gigaspora albida*) attenuated *Fusarium oxysporum* infestation of papaya seedlings (Hernández-Montiel et al. 2013). Interestingly, Sohrabi et al. (2015) demonstrated that inoculation with both *R. intraradices* and *F. mosseae* reduced *F. solani* infection in chickpeas, with *F. mosseae* being the most effective. Given that *F. mosseae* was most notable in chickpea roots and increased in response to *R. irregularis* inoculation, it could be suggested that the shift in pathogenic fungal communities observed in chickpea roots was induced by native *F. mosseae* due to the "priming" effect of *R. irregularis* inoculation.

A recent 18 S rDNA metabarcoding study also reported a negative correlation between AMF colonization and the relative frequency of *Fusarium* in *Ricinus communis* roots (Rodríguez-Yzquierdo et al. 2023). However, given that *Fusarium* is a species complex, it often is difficult to predict the functions as well as the response of the members within this group to treatments, including bioinoculation. Results from this study indicate that different species of *Fusarium* can respond differently to AMF inoculation. For instance, inoculation reduced the relative abundances of *F. nygami*, *Fusarium* sp. (identified as *Fusarium solani*), *F. tricinctum*, and *F. tokinense*, while the relative abundances of *F. hostae*, *F. equiseti*, and *F. burgessi* were positively increased. Although *F. hostae* and *F. burgessi* were increased in inoculated roots, the response of *F. equiseti* was most pronounced. The role of *Fusarium equiseti* as an endophytic fungus in plants has been controversial in the literature.

For instance, in Morocco, *F. equiseti* was recently linked to decreased seed germination and emergence, stunted growth, and root necrosis, ultimately leading to wilting in chickpea seedlings (Elhazzat et al. 2023; Adnani et al. 2024). On the contrary, *F. equiseti* also has been recognized as a beneficial plant-promoting fungus that induces systemic resistance and enhances plant growth and salt tolerance in various plant species (Feng et al. 2023). In a recent study on the mycobiome of healthy *Malva sylvestris*, ITS metabarcoding revealed *Fusarium equiseti*, along with *Alternaria subcucurbitae* as hub taxa, both were found in 80% of the samples, with *F. equiseti* being most prevalent in conditions of low precipitation (Legeay et al. 2024a). Furthermore, *A. subcucurbitae* showed a positive correlation with high potassium levels, although it was not detected in the plant roots (Legeay et al. 2024a). In the current study, both *F. equiseti* and *A. subcucurbitae* were identified in the roots of chickpea, however, *F. equiseti* exhibited a stronger response to *R. irregularis* inoculation than *A. subcucurbitae*, despite both fungal species showing a significant positive correlation with shoot biomass. Taken together, these findings suggest a potentially plant growth promoting role for these fungi, however, further studies are essential to study the nature of interaction among these fungal species *in planta*. Alternatively, it could be inferred that the increased biomass observed under *R. irregularis* inoculation stimulated root colonization by *F. equiseti* and other endophytic fungi, such as *A. subcucurbitae*. Nevertheless, isolating and characterizing these endophytes, along with investigating their potential plant growth-promoting roles in conjunction with AMF, appears promising.

Furthermore, ascochyta blight caused by *Didymella rabei* is considered to be a major economic pathogen of chickpeas in Morocco. This fungus displays a wide geographic distribution and varying aggression (Krimi Bencheqroun et al. 2022). Aggressive isolates of *D. rabiei* can cause up to 97% yield loss in chickpeas. We found two species belonging to Didymellaceae, that is, *Didymella arachidicola* (ASV266) and an unidentified *Stagnosporopsis* (ASV15 displayed a high degree of sequence similarity to *Didymella bryoniae* and *Didymella rabiei*, with an E-value of 5×10^{-174} and 100% query coverage) among the differentially abundant taxa in chickpea roots. However, Whereas *Didymella arachidicola* was enriched in AMF roots, the unidentified *Stagnosporopsis* was depleted in AMF roots (Table 1).

Isolates of *Didymella bryoniae* are commonly associated with gummy stem blight in cucurbits (cucumbers, muskmelons, and watermelons) (Stewart et al. 2015), but there is no evidence suggesting a pathogenic relation with chickpea. In contrast, *Didymella rabiei* is well documented as the causative agent of Ascochyta blight in chickpea. A few studies have previously indicated the effectiveness of AMF as effective biocontrol agents for foliar diseases, especially blights caused by *Didymella (*syn. *Ascochyta) rabiei* (Kashyap et al. 2024). For example, Moarrefzadeh et al. (2021) conducted a series of inoculation trials on two varieties of chickpea (*Saral* and *Bivanij*) and observed a remarkable suppression of *Didymella (*syn. *Ascochyta) rabiei*. *R. irregularis* had the highest disease suppression (46.15%), followed by *G. versiforme* (42.30%) and *G. fasciculatum* (40%). Therefore, the suppression of *D. rabiei* in chickpea roots through *R. irregularis* inoculation further highlights the potential of mycorrhizal inoculation as a promising practice to enhance chickpea resistance to soil-borne pathogens. However, it is equally surprising that the abundance of *D. rabiei* was positively correlated with shoot biomass data collected at the flowering stage (70 DAS). This may be because of the fungus still being in the early stages of infestation when the samples for amplicon sequencing were collected. Alternatively, the increased biomass from the higher nutritional uptake by the inoculated plants may have facilitated the colonization of *D. rabiei*.

Conclusion

We observed that *R. irregularis* inoculation increased shoot biomass but tended to decrease root biomass, as well as increasing the P, K, Na concentrations of chickpeas, while N and Zn levels remained unchanged. Additionally, fungal ITS metabarcoding revealed that *R. irregularis* inoculation notably reduced the alpha diversity of the chickpea mycobiome, particularly in the rhizosphere, but did not affect beta diversity. The findings also indicate that in addition to the promotion of nutrient uptake, the growth benefits associated with *R. irregularis* inoculation also can be attributed to the modulation of the chickpea mycobiome, resulting in the suppression of potential pathogenic fungi. This study further indicates the potential of biological solutions to address key challenges in agricultural productivity.

However, this study is not without limitations. While the root staining method indicated no effect of inoculation on root parameters, the ITS amplicon sequencing method detected some AMF taxa in chickpea roots and the rhizosphere. These findings, however, are insufficient to ascertain the persistence of *R. irregularis*, primarily due to the single point of assessment for root colonization and the non-specificity of the ITS primers for targeting AMF communities. Therefore, future long-term studies should adopt multiple sampling strategies to track the persistence and dynamics of inoculated AMF strains in the soil. Additionally, AMF-specific primers should be combined with ITS primers to encompass all fungal communities, including AMF. Furthermore, the current study did not utilize a

mock inoculum, which would have allowed us to account for the subtle effects of the inoculum medium on seedling growth. Nevertheless, the quantity and concentration of the inoculum carrier were very low, which may have limited its ability in having any significant effects. The increased biomass observed at the second harvest supports the conclusion that the chickpea response was indeed due to *R. irregularis* inoculation. Future studies are needed to investigate the responses of different local chickpea breeds to AMF inoculation in natural soils under field conditions and to unravel the molecular mechanisms responsible for pathogen suppression by AMF inoculation.

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Author contributions S.B. conducted the experiment, analyzed the data, and wrote the manuscript draft. K.A., R.E., I.K., K.E., and J.L. contributed to the methodology, data curation, review, and editing. M.H. conceived and designed the study, secured funding, supervised the work, and contributed to writing, reviewing, and editing.

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Data availability Raw reads are available in the NCBI repository under the BioProject: PRJNA1102850 and accession numbers: SAMN41030867-SAMN41030928.

Declarations

Competing interests The authors declare no competing interests.

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