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EDITED BY

Aaron S. Weed,
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REVIEWED BY

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Spanish National Research Council (CSIC),
Spain

Tingting Mei,
Zhejiang A&F University, China

*CORRESPONDENCE

Stéphane Declerck
✉ Stephan.declerck@uclouvain.be

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Paxillus involutus enhances drought resistance of *Pinus sylvestris* seedlings by improving photosynthetic rate and water use efficiency

Paola Musella^{1,2}, Ismahen Lalaymia¹, Rik Verdonck³,
Jan Colpaert³, Quentin Ponette² and Stéphane Declerck^{1*}

¹Université Catholique de Louvain, Earth and Life Institute, Mycology, Louvain-la-Neuve, Belgium,

²Université Catholique de Louvain, Earth and Life Institute, Forest Sciences, Louvain-la-Neuve, Belgium, ³University of Hasselt, Centre for Environmental Sciences (CMK), Environmental Biology Group, Diepenbeek, Belgium

Introduction: Scots pine (*Pinus sylvestris*) is an important tree species that is often regenerated through seedling establishment. The resistance of these seedlings to drought treatments and their recovery are essential to forest regeneration. The association with ectomycorrhizal (EcM) fungi such as *Paxillus involutus* has the potential to improve drought resistance and possibly recovery. Here, we conducted two experiments to evaluate the impact of *P. involutus* inoculation on growth and water use of pine seedlings under contrasting drought intensities.

Methods: In the first experiment, EcM fungal-inoculated and non-inoculated seedlings were subjected to well-watered (100% field capacity—control treatment) or low-watered (10% field capacity for 1 week—drought treatment) conditions, followed by a recovery phase in a semi-hydroponic cultivation system. In the second experiment, seedlings were initially subjected to well-watered or moderate water conditions (100 and 30% field capacity, respectively), followed by prolonged low-watered conditions (10% field capacity for 1 month), each followed by a recovery phase.

Results: In the first experiment, EcM fungal-colonized pine seedlings showed increased aboveground biomass, higher photosynthetic rates, water-use efficiency, and elevated nitrogen and phosphorus contents under low-water conditions. In the second experiment, a prolonged low-water regime reduced root colonization by the EcM fungus. During recovery, *P. involutus*-inoculated seedlings had an increase in ammonium assimilation under well-watered conditions and appeared to recover more efficiently, in particular from prolonged low-water conditions, with higher chlorophyll production, possibly due to increased nutrient availability and/or fungal demand.

Discussion: Our results suggest that the benefits of *P. involutus* depend on both water regime and drought duration: a short-term low-water regime (10% field capacity for 1 week) enhances EcM benefits, while under a moderate water regime (30% field capacity) the fungal effect was not evident. We conclude that *P. involutus* can confer significant benefits to pine seedlings, making it a valuable strategy for forest regeneration in temperate climates facing drought episodes.

KEYWORDS

ectomycorrhizal fungi, drought, seedlings, recovery, forests, climate change, drought resistance, Scots pine

1 Introduction

Global surface temperatures have risen faster since 1970 than in any other 50-year period, at least over the last 2000 years, and several regions in Europe are projected to experience an increase in the frequency and/or severity of droughts over the coming decades (IPCC, 2023). These extreme droughts combined with rising temperatures are weakening trees, threatening forest ecosystems and the services they provide. Extensive tree dieback and mortality related to drought have already been detected across the globe. For instance, over the period 2000–2022, the average annual area of European forests affected by drought was 56,000 km² (ca. 4% of forests) (Medium Resolution Vegetation Phenology and Productivity, Oct. 2022, European Environment Agency).

Drought severity is typically viewed as the primary factor leading to a decline in forest growth, although the functional traits of different tree species can also provide varying degrees of drought resistance (Greenwood et al., 2017; Peltier et al., 2016; Yin and Bauerle, 2017). Severity, duration, and frequency of drought can cause a gradual rise in tree mortality rates and even sudden die-off events. Numerous recent events of tree mortality related to drought and heat from various regions globally indicate that no forest type or climate zone is immune to the consequences of climate change, even in areas typically not considered water-limited (Allen et al., 2010). Repeated drought events cause cumulative damage to tree vascular systems, leading to increased mortality (Anderegg et al., 2015). On the other hand, severe drought may kill plants, but a mild drought or a drought for a short duration can trigger short-term memory, usually established by transcriptional training or metabolic reprogramming, leading to survival under a subsequent drought (Sadhukhan et al., 2022).

Successful seedling establishment is a prerequisite for the long-term persistence of a tree species (Lloret et al., 2009). However, seedlings are generally more sensitive to drought and less likely to recover than mature trees due to their lower carbohydrate reserves, smaller rooting volume, and shallower rooting depths than older trees (Seidel and Menzel, 2016).

P. sylvestris (Scots pine) is an ideal candidate for investigating the impacts of drought stress on seedling establishment because it is a pioneer species (Krakau et al., 2013; Vacek et al., 2021), with ecological importance and wide distribution (Pyhäjärvi et al., 2007). Research on Scots pine can provide critical insights into the mechanisms of drought tolerance, inform conservation strategies, and improve reforestation success in the face of increasing drought frequency and intensity.

Scots pine, as most temperate region forest trees, is associated with ectomycorrhizal (EcM) fungi. These root fungal symbionts may facilitate the access of seedlings to water and nutrients (Smith and Read, 1997; Lehto and Zwiazek, 2011). They are able to penetrate rock and soil pores as small as 2 µm diam thanks to the microscopic size of their hyphae (Allen, 2007), therefore accessing water and nutrients otherwise not available to roots (Lehto and Zwiazek, 2011).

Various studies have highlighted the beneficial effects of EcM fungi on tree seedlings. For example, Wang et al. (2021) showed that *Suillus variegatus* reduced the mortality rate and increased the trunk height, root and leaf biomass, photosynthetic rate, stomatal conductance and transpiration of *P. tabulaeformis* subjected to moderate to severe drought stress. Kipfer et al. (2012) also observed that *S. granulatus* can increase aboveground biomass of *P. sylvestris* seedling under drought stress conditions. Muhsin and Zwiazek (2002) found that *Hebeloma crustuliniforme* can increase root hydraulic conductivity in white

spruce (*Picea glauca*) seedlings under salt treatments. Garbaye and Churin (1997) showed that *P. involutus* significantly improved the phosphorus status of young oaks (*Quercus petraea* and *Q. robur*) during summer drought, while Danielsen and Polle (2014) showed that this EcM fungus increased potassium, magnesium and phosphorus concentrations in *Populus canescens* leaves under drought conditions.

Previous studies have shown that drought can negatively affect EcM symbioses by reducing fungal colonization and activity, particularly under prolonged or severe water stress (Davies et al., 1996; Morte et al., 2001). However, the extent of this decline is highly variable and depends on plant genotype and environmental conditions, highlighting the context-dependent nature of EcM responses to drought (Gehring et al., 2006).

While a number of studies have been conducted on the effect of EcM fungi on seedling nutrition (Colpaert et al., 1996, 1999; Zhang and George, 2010; Pohjanen et al., 2014; Nehls and Plassard, 2018), and drought stress alleviation (Bogeat-Triboulot et al., 2004; Kipfer et al., 2012; De Quesada et al., 2024; Himanen et al., 2024) fewer studies have focused specifically on the post-drought recovery phase, particularly in seedlings (Cregg and Zhang, 2001). However, recent work (Carter and Dickman, 2022; Castaño et al., 2023; Liu et al., 2024) has begun to address this gap. Understanding the effect of EcM fungi on tree seedling recovery after drought is crucial for promoting forest resilience in the face of increasing climate change. This knowledge can enhance reforestation efforts by improving seedling survival and growth in drought-prone environments.

In the present study, we investigated the effects of the EcM fungus *P. involutus* on *P. sylvestris* (Scots pine) seedlings growth under normal, moderate or low water regime and on tree recovery after drought treatments. *P. involutus* was used because it has a broad-host-range (Molina and Trappe, 1982), is very common in temperate ecosystems (Hahn, 1996) and is one of the most widely used EcM fungus in experimental laboratory work, thanks to its ease of cultivation (Wallander and Söderström, 1999). In particular, in this study, we hypothesized that *P. involutus* (1) enhances the resistance of pine seedlings to severe, moderate, and prolonged severe drought treatments (2) and improves the recovery of pine seedlings after exposure to severe, moderate, and prolonged severe drought treatments.

2 Materials and methods

2.1 Biological material

Scots pine (*P. sylvestris* L.) seeds collected in Hanau (France) were supplied by the *Comptoir Wallon des Matériels forestiers de Reproduction*. *P. involutus* (Batsch.) Fr., was supplied by the Centre for Environmental Sciences (CMK) at Hasselt University. It was obtained from basidiocarps collected in 2021 from a stand of *P. sylvestris* in Lommel (Belgium) (sampling location: 51°14'27.9564"N, 5°15'27.1188"E) and grown on Fries medium on cellophane sheets.

2.2 Experimental procedure

2.2.1 Inoculation of seedlings with *P. involutus*

Scots pine seeds were washed with Tween 20 and surface disinfected with H₂O₂ (30%). They were then sown in a substrate

made of a mixture of perlite and vermiculite (vol 2:1), previously sieved (200 μm pore size), washed with demineralized water to remove impurities and oven-dried for 48 h at 70°C. The substrate was moistened with Ingestad nutrient solution (Ingestad and Kähr, 1985) modified according to the optimum macronutrient weight proportions for *P. sylvestris* (100 N/15 P/60 K/6 Ca/6 Mg/9 S) (Nylund and Wallander, 1989). The plants were maintained in a growth chamber at 22/18°C (day/night), 65% relative humidity (RH) with a photoperiod of 12 h day⁻¹, under a photosynthetic photon flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seventy-two days after sowing, the plants reached a height of 6.0 ± 0.3 cm. The sandwich technique detailed in Colpaert et al. (1996) was used to inoculate the plants with the EcM fungus to produce mycorrhizal (M) plants. Briefly, it involved gently placing the plant roots in intimate contact with the mycelium of the EcM fungus grown on a sheet of cellophane. This arrangement was carefully nested between two layers of moist filter paper in sealed Petri dishes. Non-mycorrhizal (NM) plants followed the same procedure without adding the fungal inoculum.

2.2.2 Plant growth system

After 4 days of contact with the mycelium (for the M treatment) or without it (for NM treatment), 130 plants were transferred to inverted plastic bottles (500 mL - VWR, United States—named containers afterward) cut at the base and covered with aluminum foil to prevent the development of algae. A microporous membrane (41 μm pore size) was glued to the inside of each container at the neck to ensure the free passage of water, while preventing the passage of substrate and roots. Each container was filled with 42 g of perlite (DCM, Belgium), sieved and washed as described above. The containers were then transferred in the growth chamber (see conditions described above) on tables. On each table, five M plants, five NM plants, and two non-vegetated (NV) containers were placed and moved randomly every week. All containers were watered twice a week with Ingestad nutrient solution at field capacity.

2.2.3 Drought phases and experimental design

Eleven months after transfer to containers, 10 *P. sylvestris* seedlings—five mycorrhizal (M) and five non-mycorrhizal (NM)—were randomly selected and harvested to assess initial biomass and root colonization. The M plants showed $81.3 \pm 18.3\%$ root colonization, while no colonization was observed in NM plants. There was no significant difference in dry biomass between treatments, with M and NM seedlings averaging 1.8 ± 0.6 g and 1.9 ± 0.5 g, respectively ($p = 0.7133$). The remaining 120 plants (60 M and 60 NM) were randomly assigned to two separate drought experiments.

Experiment 1 (Figure 1A) involved 40 plants—20 M and 20 NM—divided into two water regimes. The water regimes were established by refraining from watering the containers until the soil moisture reached the desired relative water content (RWC) based on field capacity: a severe drought treatment (LWR; 10% RWC, $-1,500$ kPa) and a control treatment (NWR; 90–100% RWC, 0 kPa) (Lim et al., 2021). Once the LWR treatment reached the target RWC and was maintained for 1 week, 20 plants were harvested. The remaining 20 plants were then transferred to a semi-hydroponic recovery system for 6 days before final harvest. Once the LWR treatment reached the target RWC and was maintained for 1 week, 20 plants were harvested. The remaining 20 plants were transferred to a semi-hydroponic recovery system for 6 days before being harvested.

Experiment 2 (Figure 1B) included 80 plants—40 M and 40 NM—initially exposed to either a moderate drought treatment (MWR; 30% RWC, ~ -500 kPa) or NWR (Lim et al., 2021). After 22 days, 20 plants (five per treatment) were harvested, while the remaining 60 were transferred to the semi-hydroponic system for a six-day recovery. After recovery 20 plants were harvested. The remaining 40 plants (20 M and 20 NM) were then divided again into two groups: one continued under NWR, while the other was subjected to a prolonged severe drought treatment (PLWR; 10% RWC for 30 days, $-1,500$ kPa) (Lim et al., 2021). At the end of this prolonged drought phase, 20 plants were harvested and the remaining 20 underwent a six-day recovery period in the hydroponic system before final harvesting.

At all phases of both experiments, five biological replicates were maintained per treatment group and time point.

Water regimes were established by withholding irrigation until containers reached target RWC, monitored gravimetrically by weighing six randomly selected containers per treatment every 3 days. Field capacity, defined as the maximum water retention of 40 g of perlite, was 120 mL. Non-vegetated containers were included as evaporation controls. Plants under NWR were fertilized twice weekly with 50 mL of Ingestad nutrient solution. Moderate and severe drought treatments were simulated at 30 and 10% RWC, respectively—thresholds validated by previous studies. Meng et al. (2023) used 40 and 20% field capacity to model drought in *P. sylvestris* var. *mongolica*, and Pearson et al. (2013) reported significant drops in photochemical efficiency at 30 and 10% RWC in *P. sylvestris* seedlings. In mature trees, transpiration rates fell sharply near 12% RWC, and 10% aligns with the species' estimated permanent wilting point in sandy soils (Kirkham, 2023).

2.2.4 Recovery phase

After each period of MWR or LWR, the plants were subjected to a recovery phase, using the circulatory semi-hydroponic cultivation system developed by Garcés-Ruiz et al. (2017) (Figure 2). In brief, for each treatment, 1 L of Ingestad nutrient solution in glass bottles was circulated via peristaltic pumps through the containers and returned to the bottles in a closed circuit. After a flushing step to saturate the perlite, the inorganic P (i.e., P_i) and NH_4^+ depletion (%) in the circulating solution was measured over a period of 6 days (see for details Section 2.3.4). Initial nutrient concentration in the Ingestad solution was determined by sampling 25 mL from each bottle and considered as Time 0 (T_0). The nutrient solution was then collected as follows: the day one after 2, 4, 6, 8, and 10 h from T_0 ; the day two after 21, 24, 27, and 30 h from T_0 ; the day three after 45, 50, and 55 h from T_0 ; the day four after 70 and 80 h from T_0 ; the day five, 100 h from T_0 ; the day six, 120 h from T_0 . Finally, the plants were harvested and the solution samples were stored at 4°C in the dark.

2.3 Harvest and analysis

At each phase of Experiment 1 and 2 (Figures 1A,B), 5 plants per treatment were harvested. Just before harvesting, physiological indicators such as leaf gas exchange and chlorophyll content were measured. Stems were then cut to determine shoot water potential and plants harvested entirely to assess morphological characteristics.

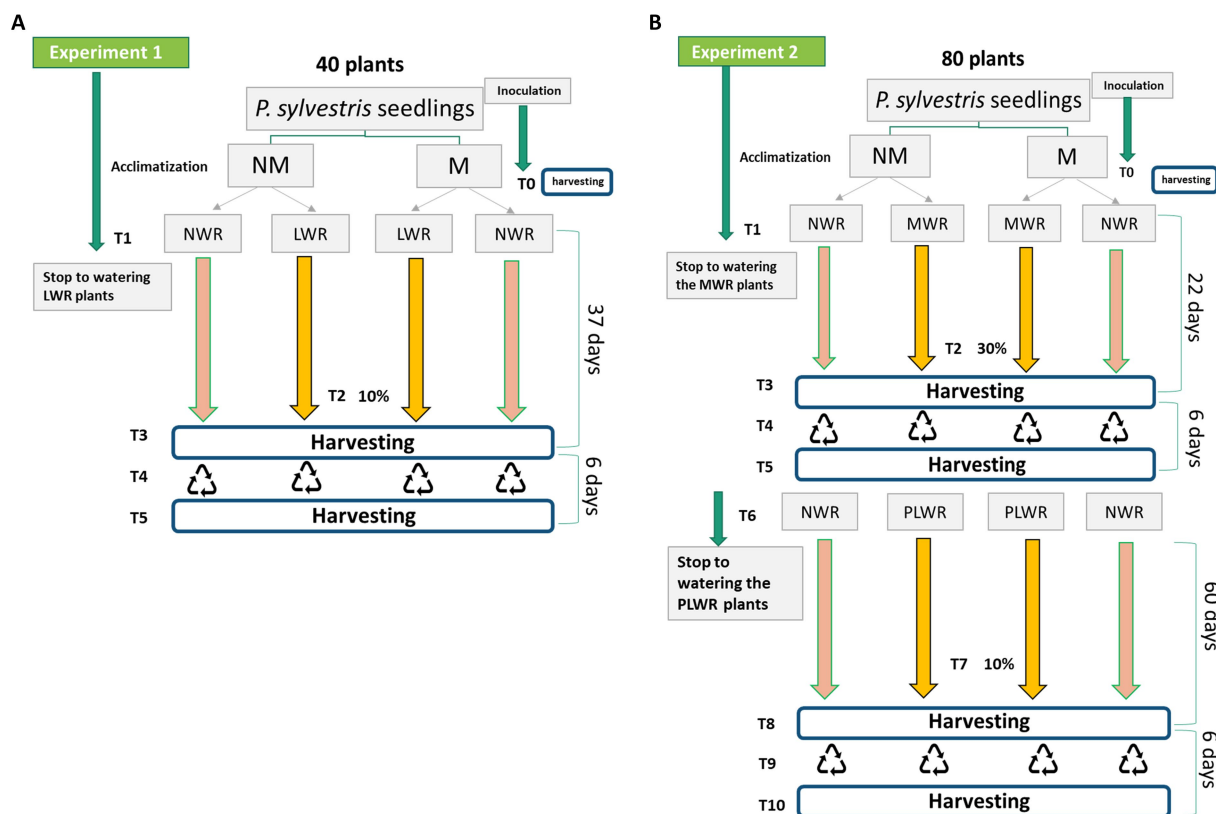


FIGURE 1

(A) Schematic representation of the experimental design for Experiment 1 and (B) for Experiment 2. NM = non-mycorrhizal plants; M = mycorrhizal plants; NWR = normal water regime (i.e., the control); LWR = low water regime (watering reduced and maintained to 10–15% RWC for 1 week); MWR = moderate water regime (watering reduced to 30–35% RWC); PLWR: prolonged low water regime (watering reduced during 30 days and maintained to 10–15% RWC for one more month). *Experiment 1*: T0 = Fungal inoculation; T1 = interruption of watering the LWR treatments; T2 = Containers of the LWR treatments reaching 10–15% RWC; T3 = harvesting of 20 plants (5 per treatment); T4 = Start of water circulation for 20 containers for 1 week; T5 = harvesting of 20 plants (5 per treatment). *Experiment 2*: T0 = Fungal inoculation; T1 = Interruption of watering the MWR treatments; T2 = Containers reaching 30–35% RWC; T3 = harvesting of 20 plants (5 per treatment); T4 = Start of water circulation for 60 containers for 1 week; T5 = harvesting of 20 plants (5 per treatment); T6 = Interruption of watering the PLWR treatments; T7 = Containers reaching 10% RWC, kept for 1 month; T8 = harvesting of 20 plants (5 per treatment); T9 = Start of water circulation for 20 containers (5 per treatment) for 1 week; T10 = harvesting of 20 plants.

2.3.1 EcM fungal colonization of root

Root colonization rate (RCR) was estimated on three randomly selected root sub-samples per plant. The RCR was obtained by counting the number of tips colonized by the EcM fungus divided by the total number of tips observed (Brundrett et al., 1996).

2.3.2 Physiological parameters

Just before harvesting, photosynthetic rate, stomatal conductance, and transpiration rates were measured using an integrated fluorometer and gas exchange system (ADC Bioscientific Ltd., Hoddesdon, United Kingdom), with the application of LCpro-SD, a portable conifer-specific photosynthesis measurement chamber. Gas exchange measurements were performed between 9:00 and 18:00 under controlled chamber conditions with constant light intensity of $999 \mu\text{mol m}^{-2} \text{s}^{-1}$, chosen based on a preliminary light response curve. CO_2 reference concentrations ranged from 427 to 452 ppm, and humidity inside the needle chamber (H_2O of reference) ranged from 9.9 to 10.8 mmol mol^{-1} . Instantaneous water-use efficiency (WUE_i) was calculated using the equation of Bacelar et al. (2012): $\text{WUE}_i = \text{Photosynthetic rate} / \text{Transpiration rate}$.

The chlorophyll content of the needles was measured on four needles per plant before and after recovery phase on the same plants (5 per treatment), using a Chlorophyll Content Meter adapted for very small leaves (CCM-300, Opti-Sciences, Inc., USA), which uses a fluorescence ratio technique for chlorophyll content measurement according to Gitelson et al. (1999). The difference between the values before and after the recovery phase was then calculated.

Shoot water potential (Ψ) was measured at midday (between 12:00 and 14:00) under full light conditions, in order to assess plant water status during peak transpiration. Shoots were excised at the base of the stem immediately before the measurement. Water potential was then determined using an airtight pressure chamber (Model 3,115, Soil Moisture Equipment Corp., USA), in which xylem sap is forced out of the plant tracheids by increasing pressure in the chamber, following the method of Scholander et al. (1965).

2.3.3 Plant growth parameters

The height of the main stem was measured, followed by fresh weight of shoots and roots. The volume of the roots was calculated in a graduated cylinder, measuring the difference between the water volume before and after adding the roots to the cylinder.

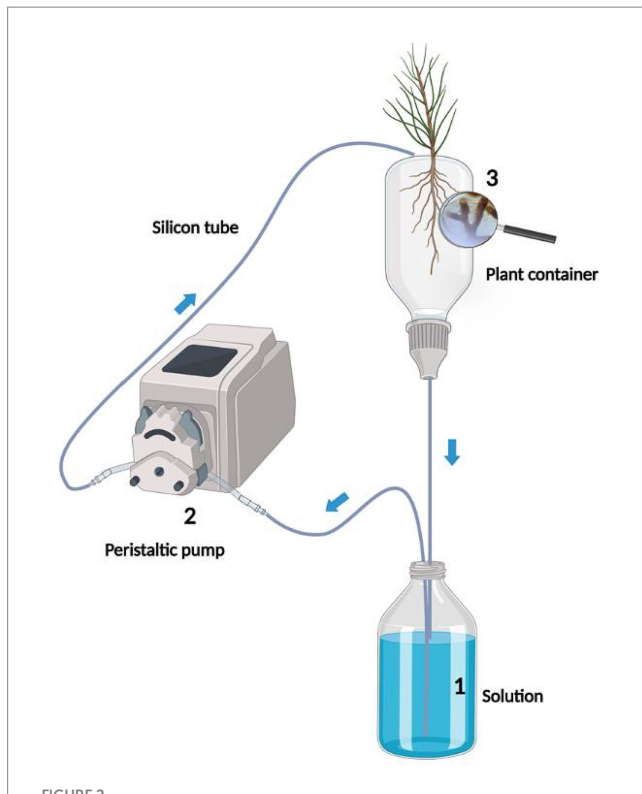


FIGURE 2

Schematic representation of the circulatory semi-hydroponic cultivation system, used to inorganic P (Pi) and NH_4^+ depletion in the ingested solution circulating through the containers. The nutrient solution in the glass bottle (1) is pumped with a peristaltic pump (2) to the upper part of the container (3) via silicon tubes. The solution percolates through the plant container back into the glass bottle. Gray arrows indicate the flow direction of the nutrient solution in the tubing (Garcés-Ruiz et al., 2017; Cartabia et al., 2021).

Roots and needles of each plant were then stored at -80°C and freeze-dried to obtain dry weights. The water content of the roots and shoots was expressed as g of H_2O on g of biomass. After freeze-drying, the roots and shoots samples were ground separately to powder using a mortar and liquid nitrogen, and the concentrations (%) of phosphorus (P), carbon (C), and nitrogen (N) were determined using an inductively coupled plasma atomic emission spectroscopy (ICP-AES), after acid digestion of tissues with HNO_3 (ISO 11466:1995) for P and an Elemental Analyzer System (Nelson and Sommers, 1996) for C and N. Phosphorus, C and N content of shoots and roots were calculated by multiplying the nutrient concentration by the dry biomass of shoots and roots.

2.3.4 Measurement of nutrient uptake in the semi-hydroponic cultivation system

The P_i concentration was measured by ICP-AES as described in Garcés-Ruiz et al. (2017). Data were then normalized to the NV controls (Garcés-Ruiz et al., 2017) and converted to % concentrations of P_i in the nutrient solution relative to the initial concentration at T_0 , using the following formula:

$$\%(Px) = \left(\frac{[Px]T - [PNV]T - [PNV]T_0}{[Px]T_0} \right) \times 100$$

where:

$[P]$ = P_i concentration in the solution.

x = sample.

NV = non-vegetated containers (i.e., perlite control).

$[P_{NV}]$ = mean P_i concentration for the NV containers.

T = time considered

T_0 = start of the circulatory system.

For NH_4^+ , the concentration was determined by colorimetric analysis, using the Merck Spectroquant Ammonium-Test (1.14752, Merck - Darmstadt, Germany) which uses the transformation reaction of ammonium into blue indophenol. The same formula as above was applied to ammonium concentration.

2.4 Statistical analysis

Data analysis was performed with JMP Pro 17 with an α -threshold of 5%. Levene and Shapiro-Wilk tests were performed before statistical analysis to confirm the homogeneity of variance and the normality of distribution, respectively. The effects of the factors mycorrhization (Myc) and water regime (WR) were investigated using a general linear model (GLM) followed by a pairwise comparison using the Tukey-HSD test.

3 Results

3.1 Experiment 1

The following sections describe the effects of water regime WR (low water regime, LWR, and normal water regime, NWR) and mycorrhization (Myc) on root colonization, plant growth, physiological parameters, plant nutrient status, and recovery phase of *P. sylvestris* seedlings.

3.1.1 Root colonization

Whatever the WR, root colonization was observed in each plant, with typical dichotomous tips surrounded by extraradical hyphae (Figure 3). Root colonization rates were unaffected with values of $99.7 \pm 0.3\%$ and $92.5 \pm 5.9\%$, for the NWR and LWR treatments, respectively. No root colonization was observed in the plants of the NM treatments.

3.1.2 Plant growth

Total plant biomass (i.e., shoot + roots) was significantly affected by the factor Myc ($F_{1, 16} = 3.23$, $p < 0.01$) and the factor WR ($F_{1, 16} = 2.90$, $p < 0.05$). No interaction effect was observed between both factors. Pairwise comparisons showed that the total plant biomass in the NM-LWR treatment was significantly lower than that of the plants in the M-NWR treatment. No other differences were observed among the treatments (Figure 4A). A major impact of the factors Myc and WR was observed on the aboveground biomass of the plants of the NM-LWR treatment ($F_{1, 16} = 10.4$, $p < 0.001$, $F_{1, 16} = 8.42$, $p < 0.005$, respectively), with values significantly lower than that of all other treatments (Figure 4B). The LWR impacted aboveground biomass but not belowground biomass (Figure 4C). The factor Myc significantly impacted the root/shoot ratio ($F_{1, 16} = 6.11$, $p < 0.05$), while the factor WR had no significant effect on this parameter. Pairwise comparisons



FIGURE 3
Characteristic dichotomous root tips induced by *P. involutus* in *P. sylvestris* seedlings (black double arrows), surrounded by mycelium (white arrows). Scale bar = 1 μ m.

showed that the plants grown in the M-NWR and M-LWR had a significantly lower root/shoot ratio than those grown in the NM-LWR treatment (Figure 4D). An ANCOVA was performed to assess the effect of treatment on the root/shoot ratio with total biomass as covariate. The analysis showed that treatment had a significant effect ($p < 0.05$), while total biomass and its interaction with treatment were not significant ($p > 0.05$), indicating that treatment differences in root/shoot ratio were independent of plant size.

3.1.3 Plant physiology

The photosynthetic rate was significantly affected by the factor Myc ($F_{1,16} = 27.2$, $p < 0.0001$) and the factor WR ($F_{1,16} = 6.20$, $p < 0.05$). No significant effect was observed for the interaction Myc \times WR. Pairwise comparison showed that the plants of the NM-LWR treatment had a significantly lower photosynthetic rate than the plants of the M treatments (Figure 5A) but did not differ from the plants in the NM-NWR treatment. Similarly, transpiration rate was significantly affected by the factor Myc ($F_{1,16} = 5.90$, $p < 0.05$) and the factor WR ($F_{1,16} = 6.00$, $p < 0.05$). Pairwise comparison showed that the plants of the NM-LWR treatment had a significantly lower transpiration rate than the plants of the M-NWR treatment (Figure 5B). Water use efficiency was significantly impacted by the factor Myc ($F_{1,16} = 30.0$, $p < 0.0001$) but not by the factor WR. The interaction between the two factors was also significant ($F_{1,16} = 6.20$, $p < 0.05$). Pairwise comparison showed that the plants of the NM-LWR treatment had a significantly lower water use efficiency compared to those in the M-LWR and M-NWR treatments, but did not differ from the plants in the NM-NWR treatment. In addition, plants in the M-LWR treatment had significantly higher water use efficiency than both the NM treatments (Figure 5C). The other physiological parameters (i.e., stomatal conductance, water potential) did not differ significantly between the treatments (Supplementary material S1).

3.1.4 Plant nutrient status

Nitrogen, P and C concentrations of plants were not significantly impacted by the factors Myc and WR (Figure 6). Conversely, the N content of plants was significantly impacted by the factor Myc ($F_{1,16} = 4.80$, $p < 0.05$), the factor WR ($F_{1,16} = 5.00$, $p < 0.05$) and their

interaction ($F_{1,16} = 6.90$, $p < 0.05$). Pairwise comparisons showed that N content was significantly lower in the plants of the NM-LWR treatment compared to the plants in the other treatments (Figure 7A). Plant P content was also affected by the factor Myc ($F_{1,16} = 18.2$, $p < 0.001$) and WR ($F_{1,16} = 4.70$, $p < 0.05$) but not by their interaction. The total P content in the plants of the NM-LWR treatment was significantly lower compared to that of the plants in the M treatments but did not differ from those in the NM-NWR treatment (Figure 7B). The total C content of the plants was also significantly impacted by the factor Myc ($F_{1,16} = 10.7$, $p < 0.01$) and the factor WR ($F_{1,16} = 7.30$, $p < 0.05$). No effect of the interaction between factors Myc \times WR was found for this parameter. Pairwise comparison showed that the total C content of the plants of the NM-LWR treatment was significantly lower compared to that of the plants in the M-NWR treatment, but did not differ from the two other treatments (Figure 7C).

3.1.5 Plant recovery

At the end of the recovery phase, the colonization rate did not differ significantly between the plants in the M-LWR treatment and those in the M-NWR treatment. The percentage of mycorrhization was $78.9 \pm 8.3\%$ and $91.2 \pm 5.4\%$, respectively. No root colonization was observed in the plants of the NM-NWR and NM-LWR treatments.

Plant growth and physiological parameters were not affected by the factor Myc, while the factor WR affected significantly the photosynthetic rate ($F_{1,16} = 4.60$, $p < 0.05$), the transpiration rate ($F_{1,16} = 5.30$, $p < 0.05$), stomatal conductance ($F_{1,16} = 4.70$, $p < 0.05$) with a decrease of 24.1, 17.5 and 19.8%, respectively, in plants of the LWR treatment compared to those in the NWR treatment. Water use efficiency was not affected by neither factor (Supplementary material S2). In plants subjected to LWR, the difference in chlorophyll content before and after recovery was not significantly impacted by the factors Myc and WR (Supplementary material S2).

3.2 Experiment 2

3.2.1 First cycle of drought treatment

The following sections describe the effects of water regime WR (moderate water regime, MWR, and normal water regime, NWR) and mycorrhization (Myc) on root colonization, plant growth, physiological parameters, and recovery phase of *P. sylvestris* seedlings.

3.2.1.1 Root colonization

Root colonization rates were not affected by the WR. For the mycorrhizal plants grown under NWR and MWR, the rate of root colonization was $64.2 \pm 18.1\%$ and $54.3 \pm 15.0\%$, respectively. No root colonization was observed for the plants in the NM treatments.

3.2.1.2 Plant growth

The factor Myc and WR and their interaction did not impact any of the growth parameters (Supplementary material S3).

3.2.1.3 Physiological parameters

The photosynthetic rate was not significantly impacted by the factor WR and the factor Myc. Transpiration rate was significantly affected by the factor WR ($F_{1,16} = 24.5$, $p < 0.0001$) and by the factor Myc ($F_{1,16} = 8.13$, $p < 0.01$) but not by their interaction. Pairwise comparison showed that plants of the MWR treatments had a

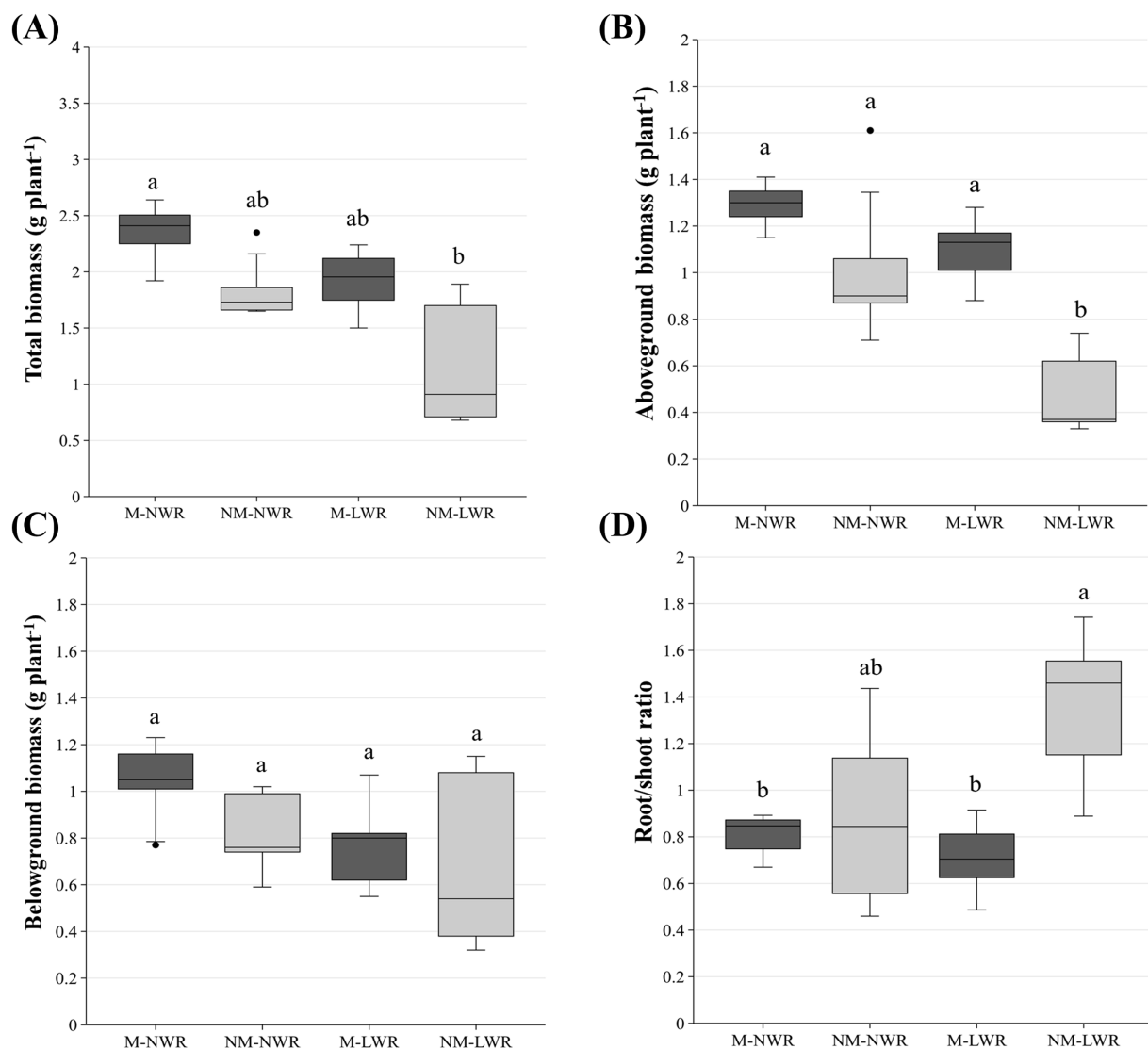


FIGURE 4

Exp 1: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on (A) total plant biomass (i.e., shoot + roots); (B) on aboveground biomass, (C) on belowground biomass and (D) on root/shoot ratio. The box plot displays the interquartile range (IQR) of the scores (central boxes), with the lower edge of the box indicating the first quartile and the upper edge marking the third quartile. The horizontal line inside each box represents the median score. The whiskers extend to the smallest and largest values within $1.5 \times \text{IQR}$ from the first and third quartiles. Scores outside this range are considered outliers and are depicted as individual points. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. LWR: low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

significantly lower transpiration rate compared to those in the M-NWR treatment, but did not differ from the plants in the NM-NWR treatment. Stomatal conductance was also significantly affected by the factor WR ($F_{1,16} = 19.7$, $p < 0.0005$), with the plants in the MWR treatments showing a significant difference with the plants in M-NWR treatment. Pairwise comparison did not show any significant effects on water use efficiency and water potential (Supplementary material S3).

3.2.1.4 Plant recovery

The colonization rate was not impacted by the factor WR. Root colonization of the plants in the M-NWR and M-MWR treatments was $75.9 \pm 18.9\%$ and $62.5 \pm 21.7\%$, respectively. No root colonization was observed in the plants of the NM treatments.

The factor *Myc* had a significant effect on photosynthetic rate, transpiration rate, and water use efficiency ($F_{1,16} = 15.2$, $p < 0.001$; $F_{1,16} = 8.10$, $p < 0.01$; $F_{1,16} = 6.70$, $p < 0.05$, respectively), while stomatal conductance was not affected. The factor WR significantly affected the photosynthetic rate and transpiration rate ($F_{1,16} = 7.70$, $p < 0.05$; $F_{1,16} = 24.5$, $p < 0.0001$). Pairwise comparison showed that photosynthetic rate was significantly higher in the plants of M-NWR treatment compared to the other treatments, while transpiration rate was significantly higher compared to MWR treatments but not compared to the NM-NWR treatment (Figure 8).

Pairwise comparison did not show any significant effects on the growth and the other physiological parameters (Supplementary material S4).

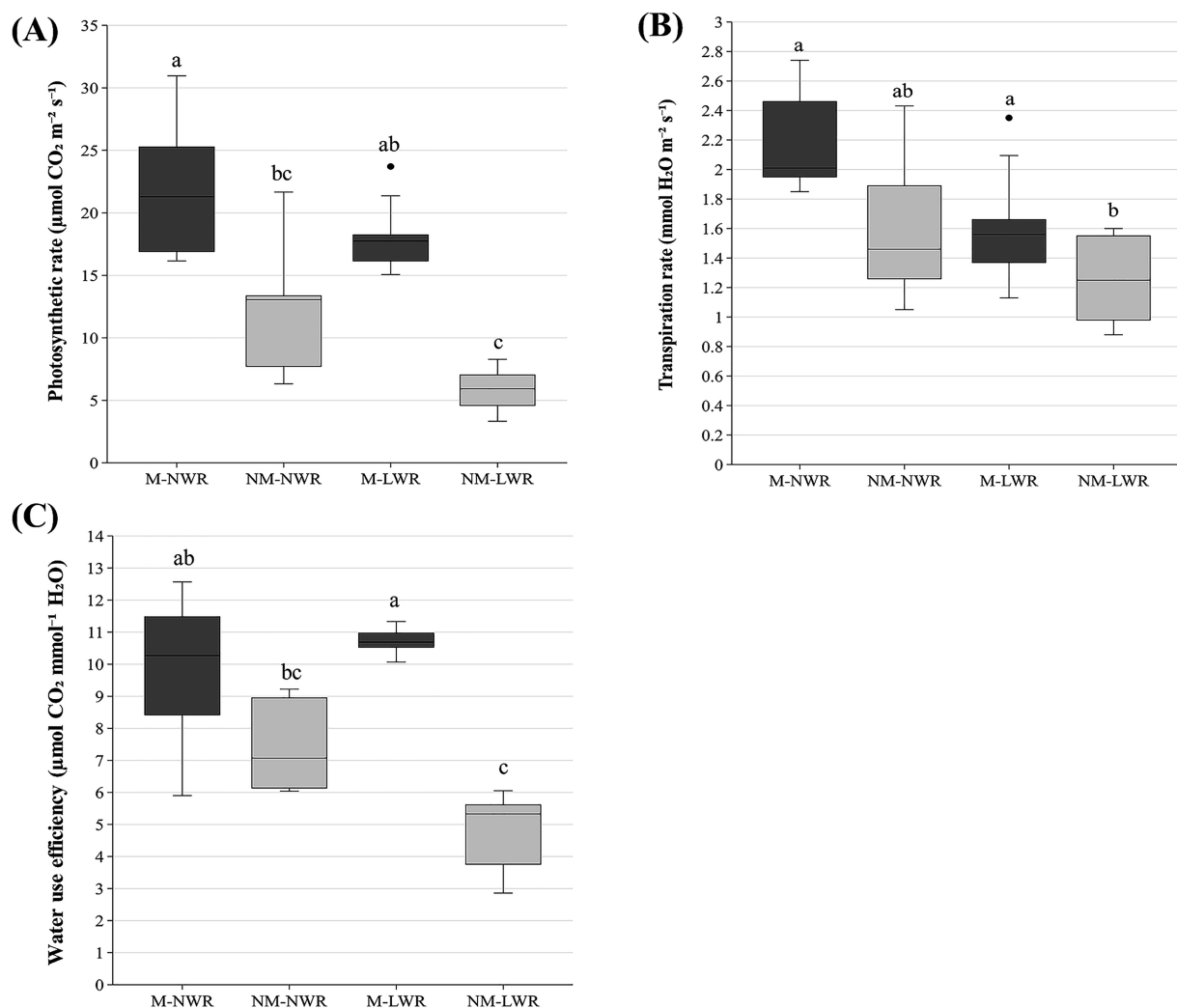


FIGURE 5

Exp 1: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on (A) photosynthetic rate (B) transpiration rate and (C) water use efficiency. The box plot displays the IQR of the scores, with the lower edge of the box indicating the first quartile and the upper edge marking the third quartile. The horizontal line inside each box represents the median score. The whiskers extend to the smallest and largest values within $1.5 \times \text{IQR}$ from the first and third quartiles. Scores outside this range are considered outliers and are depicted as individual points. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. LWR: low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

During the recovery phase, no significant difference was observed between the treatments in the depletion of P_i (Supplementary material S4). Conversely, The factor *Myc* ($F_{1,16} = 31.7$, $p < 0.0001$), WR ($F_{1,16} = 21.5$, $p < 0.0001$), and their interaction ($F_{1,16} = 29.3$, $p < 0.0001$) had a significant effect on the depletion of NH_4^+ at D3T3. Pairwise comparisons showed a significant lower value of NH_4^+ concentration in the solution in plants of M-NWR treatment at D3T3 compared to the other treatments (Figure 9).

In plants subjected to MWR, the difference in chlorophyll content before and after recovery was not significantly affected by the factors *Myc* and WR (Supplementary material S4).

3.2.2 Second cycle of drought treatment

The following sections describe the effects of water regime WR (prolonged low water regime, PLWR, and normal water regime,

NWR) and mycorrhization (*Myc*) on root colonization, plant growth, physiological parameters, and recovery phase of *P. sylvestris* seedlings.

3.2.2.1 Root colonization

Root colonization was significantly affected by WR ($p = 0.0316$). It was significantly higher for the plants in the M-NWR treatment (i.e., $94.2 \pm 9.5\%$) compared to the plants in the M-PLWR treatment (i.e., $63.7 \pm 22.1\%$). No root colonization was observed for the plants in the NM treatments.

3.2.2.2 Plant growth

Total biomass was significantly impacted by the factor *Myc* ($F_{1,16} = 6.70$, $p < 0.05$) but not by the factor WR. No interaction effect between both factors was observed. Pairwise comparison showed that the biomass of plants in the NM-LWR treatment was significantly lower than in those of the M-NWR treatment (Figure 10). No other

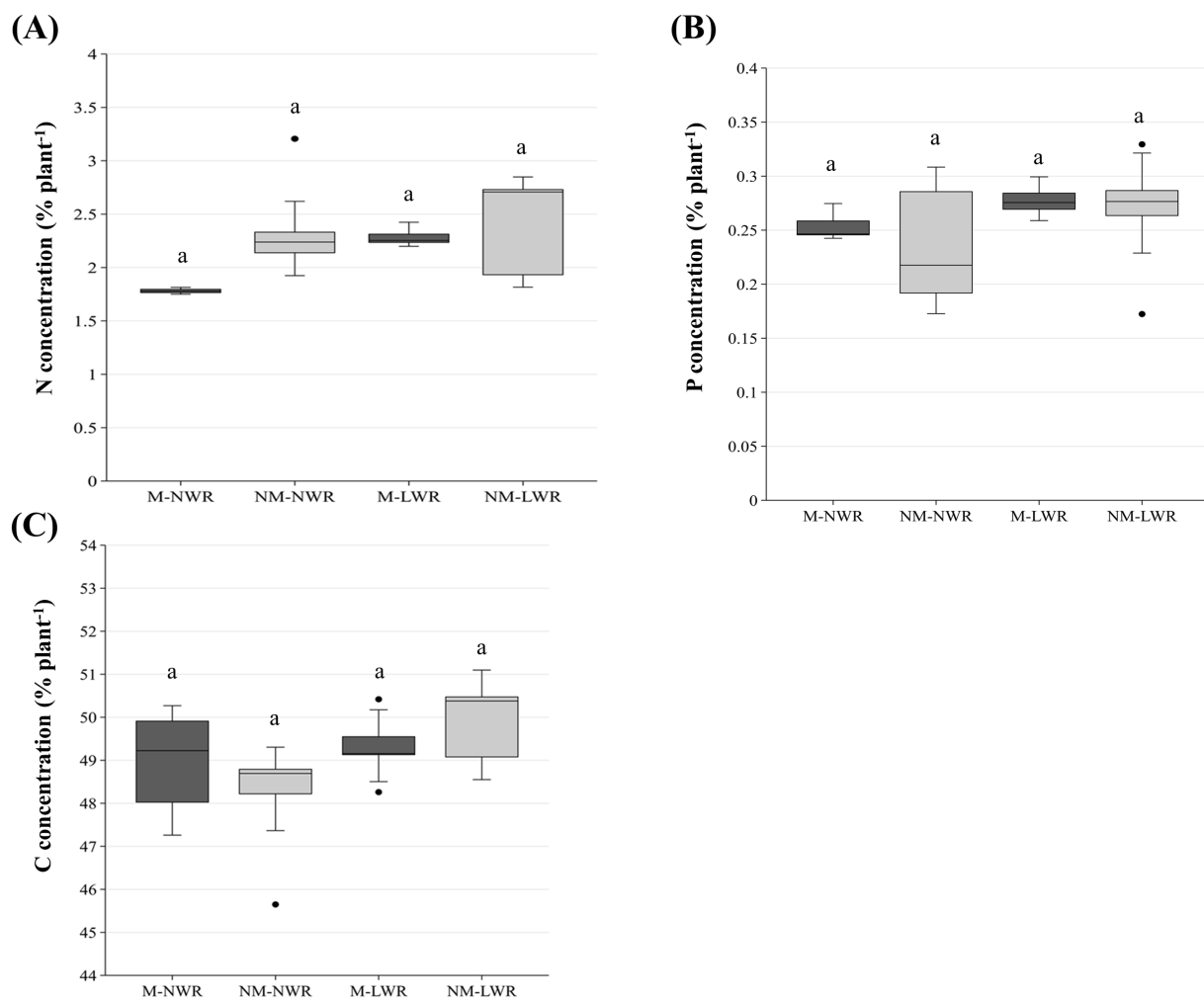


FIGURE 6

Exp 1: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on concentrations of (A) nitrogen (N), (B) phosphorus (P) and (C) carbon (C). The height of each bar indicates the average concentration of the respective element for that treatment. Error bars on top of each bar represent the standard deviation. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. LWR: low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

growth parameters (i.e., fresh weight, root volume, shoot height) differed between the treatments (Supplementary material S5).

3.2.2.3 Physiological parameters

Physiological parameters photosynthetic rate, transpiration rate and stomatal conductance were significantly affected by the factor WR ($F_{1,16} = 10.2$, $p < 0.01$), ($F_{1,16} = 30.2$, $p < 0.0001$), ($F_{1,16} = 19.4$, $p < 0.001$), but water use efficiency was not affected by WR. The factor Myc did not significantly affect these parameters. Pairwise comparisons showed that plants in the NM-PLWR had a significantly lower photosynthetic rate compared to the M-NWR treatment and significantly lower transpiration rate and stomatal conductance compared to the NWR treatments (Figures 11A–C).

3.2.2.4 Plant recovery

The colonization rate was significantly impacted by the factor WR ($p < 0.01$) with values significantly higher for the plants in the M-NWR treatment ($90.8 \pm 13.8\%$) compared to the plants in the

M-PLWR treatments ($40.0 \pm 14.7\%$). No root colonization was observed for the plants in the NM treatments.

Plant growth parameters were not affected by the factors Myc, WR or their interaction, during recovery phase and did not show significant differences between the treatments (Supplementary material S6).

The photosynthetic rate was significantly affected by the factor Myc ($F_{1,16} = 8.50$, $p < 0.01$) and by the factor WR ($F_{1,16} = 15.5$, $p < 0.001$), but not by their interaction. Pairwise comparison showed that the photosynthetic rate in plants of the NM-PLWR treatment was significantly lower than those in the M-NWR treatment, while no other differences were observed. Similarly, stomatal conductance and water use efficiency were significantly affected by the factor WR ($F_{1,16} = 10.6$, $p < 0.01$ and $F_{1,16} = 8.20$, $p < 0.05$, respectively) but not by the factor Myc. No interaction between both factors was observed. The other physiological parameters (i.e., transpiration rate, and water potential), did not show a significant effect (Supplementary material S6).

The chlorophyll content was significantly influenced by the factor Myc ($F_{1,16} = 32.5$, $p < 0.0001$), but not by the factor WR, although their

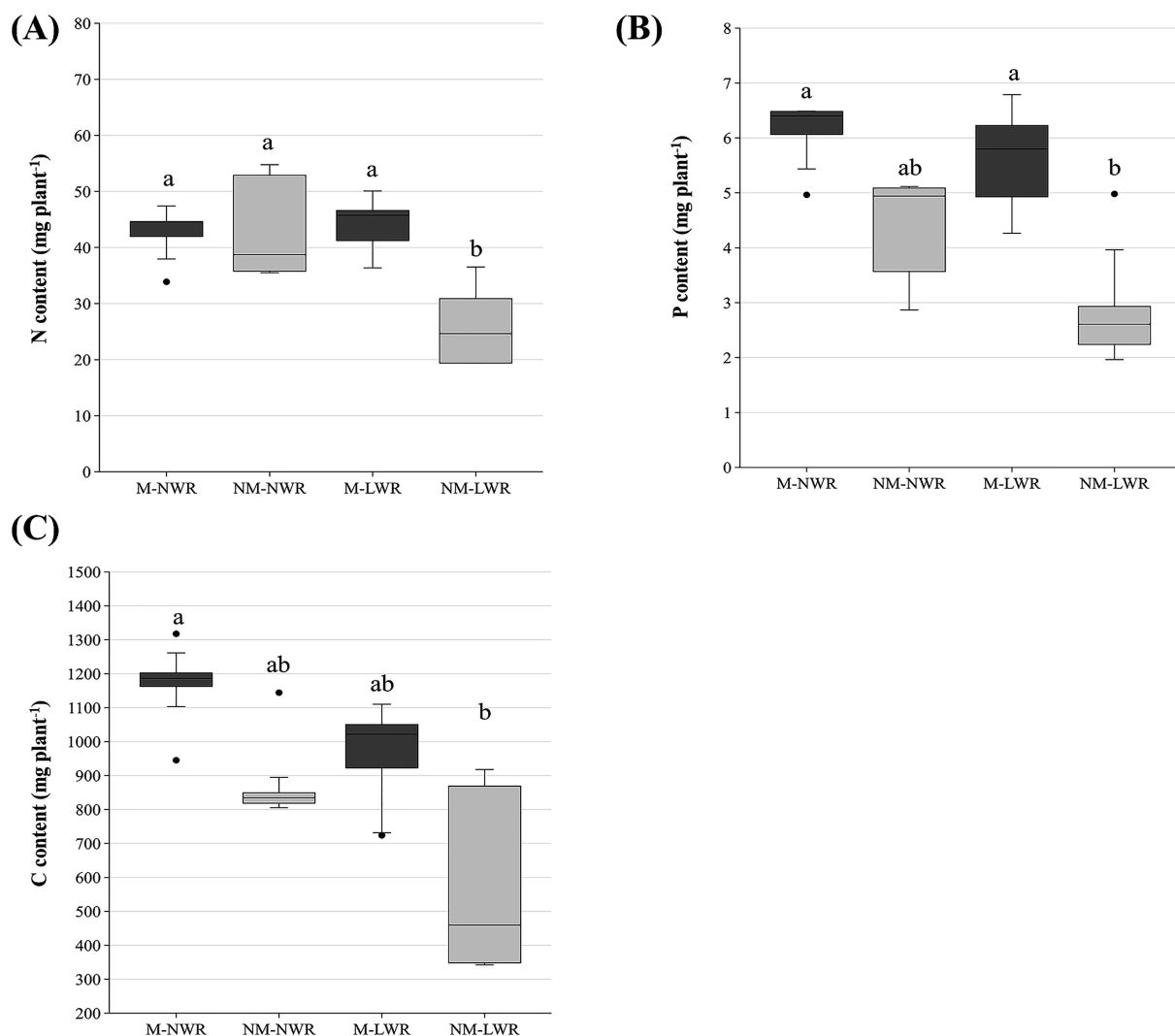


FIGURE 7

Exp 1: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on contents of (A) nitrogen (N), (B) phosphorus (P) and (C) carbon (C). The height of each bar indicates the average content of the respective element for that treatment. Error bars on top of each bar represent the standard deviation. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. LWR: low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

interaction was significant ($F_{1,16} = 32.2$, $p < 0.0001$). Pairwise comparison showed a significant decrease in chlorophyll content in plants of the NM-PLWR treatment compared to those in the NWR treatment, while the plants in the M-PLWR treatment showed a significant increase (Figure 12).

4 Discussion

4.1 Effect of *P. involutus* on seedlings growth and physiological parameters under contrasting water regimes

Ectomycorrhizal fungi have been widely studied for their contributions to forest regeneration, mainly because of their potential to enhance the growth and survival of trees under adverse conditions (Brundrett, 2009). However, studies of how different drought

treatments affect tree seedlings inoculated with EcM fungi have produced mixed results, often showing variability depending on fungal and tree species, plant's age and environmental context.

Here, we compared the growth and physiology of *P. sylvestris* seedlings, associated or not to the EcM fungus *P. involutus*, under different drought treatments (i.e., normal water regime (NWR), moderate water regime (MWR), low water regime (LWR), and prolonged low water regime (PLWR)), and during recovery from drought treatments. We have shown that mycorrhizal plants exhibit increased aboveground biomass, photosynthetic rate and water use efficiency, particularly under LWR.

4.1.1 Low water regime

Under LWR conditions, the total plant biomass (shoot + roots) and shoot biomass of seedlings associated with the EcM fungus was significantly higher compared to the non-colonized ones, while no difference was observed for root biomass (Figures 4A–C). This

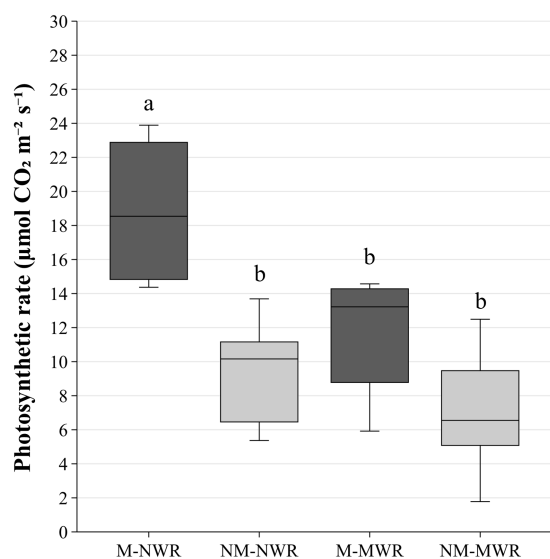


FIGURE 8

Exp 2: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on photosynthetic rate after recovery phase. The box plot displays the IQR of the scores, with the lower edge of the box indicating the first quartile and the upper edge marking the third quartile. The horizontal line inside each box represents the median score. The whiskers extend to the smallest and largest values within 1.5 * IQR from the first and third quartiles. Scores outside this range are considered outliers and are depicted as individual points. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. MWR: moderate water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

increase in shoot biomass suggests that the plants associated to the EcM fungus allocated more resources to aboveground parts. This result corroborates the study of Yin et al. (2018) who explored how *P. sylvestris* var. *mongolica* seedlings inoculated with *S. luteus* responded to water stress. They found that EcM fungal-inoculated seedlings exhibited significantly greater growth than their non-inoculated counterparts. Similarly, Li et al. (2023) demonstrated that *P. massoniana* seedlings inoculated with *Suillus luteus* had significantly higher aboveground biomass, but in contrast to our study, also higher root biomass, under conditions of moderate and severe drought treatments. This was also reported by Wang et al. (2021), who observed that the inoculation of *P. tabulaeformis* seedlings with *S. variegatus* resulted in a marked increase in leaf biomass and as well root biomass, all under conditions of moderate and severe drought treatments. This suggests that EcM fungal inoculation can provide a buffer against drought, helping seedlings to maintain growth and development under limited water conditions. Results may also vary according to the EcM fungus as shown by Kipfer et al. (2012). These authors discovered that among eight EcM fungal species tested, only *S. granulatus* had a significant positive effect on the shoot growth of *P. sylvestris* seedlings because it was the fungus with the highest activity of secreted extracellular enzymes. This beneficial effect was more pronounced under well-watered conditions compared to dry conditions, suggesting that EcM fungi may not always provide additional support during periods of drought. This variability in EcM fungal effectiveness could be attributed to the fungal species, the

extent of colonization, and the environmental context in which the symbiosis occurs.

When comparing the root-to-shoot ratio, we noticed that it was significantly higher in the non-colonized seedlings under LWR (Figure 4D), suggesting that these plants allocated more resources to root growth, potentially as a strategy to access limited water resources. However, this allocation came at the expense of aboveground parts, indicating a trade-off between root and shoot growth under drought conditions. Moser et al. (2015) also emphasized the importance of root architecture plasticity in *P. sylvestris* seedlings during early establishment under drought conditions. Their results suggested that the structural adaptability of the root system played a more crucial role in the seedlings' drought resistance than colonization by EcM fungi. This supports the hypothesis that while EcM symbiosis can be beneficial, other factors such as the inherent plasticity in biomass allocation patterns may also play an important role in plant survival under stress.

The resource allocation pattern in NM-LWR seedlings (where resources are allocated in a way that prioritizes root biomass over aboveground growth) could also be hypothetically explained by the reduced photosynthetic rate observed. Indeed, the NM-LWR seedlings showed a 63.8% decrease in photosynthesis compared with the M-LWR seedlings (Figure 5A). Colpaert et al. (1996) also found that EcM fungi stimulated net photosynthesis in *P. sylvestris* seedlings colonized with *S. bovinus*, but in this case this increase did not translate into greater shoot growth and failed to compensate for the costs of maintaining the symbiotic relationship. Kipfer et al. (2012), did not investigate photosynthetic activity but in contrast reported that EcM fungal inoculation increased aboveground biomass in *P. sylvestris* seedlings, but only when inoculated with *S. granulatus*. In presence of *P. involutus*, *Cenococcum geophilum* or *Rhizopogon roseolus*, no significant increase in aboveground biomass was observed under either dry or wet conditions. This suggests that the benefits of EcM fungal inoculation is highly species-specific.

Under LWR, the photosynthetic rate of non-EcM fungus-associated seedlings was significantly negatively affected. In contrast, in presence of the EcM fungus, the seedlings exhibited no significant differences in photosynthesis compared to the well-watered treatments. This indicated that the association with the EcM fungus helped the seedlings to maintain photosynthetic efficiency under drought treatment conditions. Furthermore, we observed that the transpiration rate and WUE_i differed significantly between the M and NM seedlings under LWR (Figures 5B,C). However, neither group exhibited differences from their respective well-watered controls, suggesting that the EcM fungus colonized seedlings maintained a higher WUE_i across different water regimes and M-LWR seedlings maintained an even higher WUE_i compared to NM seedlings in the control group (Figure 5C). This could be explained by the demand of EcM fungi for carbon, which in turn promotes photosynthesis (Lamhamedi et al., 1994). As a consequence of the increased carbohydrate demand in symbiosis, trees increase their photosynthetic capacity (Nehls et al., 2010). Similarly, Li et al. (2023) also highlighted the role of *S. luteus* in improving the plant's hydraulic regulation, particularly by increasing WUE and promoting a more balanced water status in the plant.

On the other hand, the fact that the non-inoculated seedlings showed a reduced photosynthetic rate and aboveground biomass, could be hypothetically attributed to the isohydric behavior of

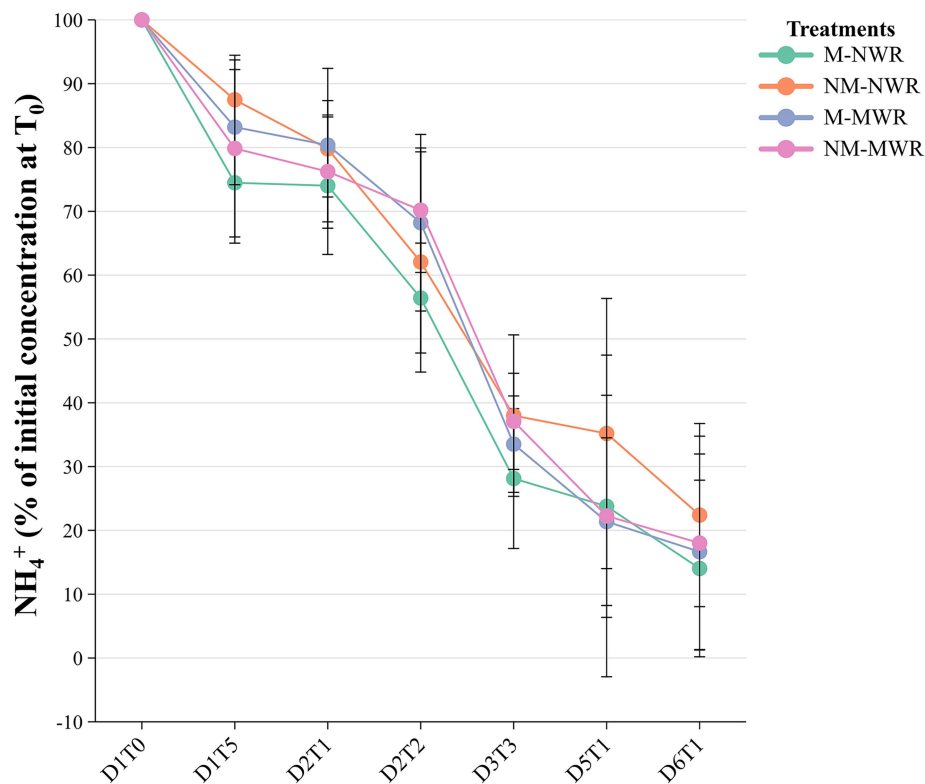


FIGURE 9

Exp 2: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on the depletion of ammonium during recovery phase. Data ($n = 8/12$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$) per each day and timing. MWR: moderate water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants; D = day of sampling; T = time of sampling during the day.

P. sylvestris, characterized by strict stomatal control that limits transpiration when soil moisture falls below a certain threshold (Irvine et al., 1998; Poyatos et al., 2007; Martínez-Vilalta et al., 2009), even if data of stomatal conductance and water potential in the present work do not truly validate this hypothesis. It has also to be considered that this isohydric strategy, while helping to conserve water, also limits carbon assimilation (Martín-Gómez et al., 2017).

When analyzing the nutrient status of the seedlings subjected to NWR and LWR, we found that the concentrations of N and P did not differ significantly between the seedlings associated or not to the EcM fungus (Figures 6A,B). However, the N content in NM-LWR seedlings was 39.5% lower compared to M-LWR seedlings (Figure 7A). This difference in N content is directly linked to the increased shoot biomass in the M-LWR seedlings. Similar differences were observed in P content, with NM-LWR seedlings exhibiting 46.3% lower P content compared to M-LWR seedlings, while no significant differences were observed between NM-LWR and NM-NWR seedlings (Figure 7B). The inoculated plants in the LW treatment received less water and nutrients than those in the NW treatment. Nevertheless, they absorbed similar amounts of nutrients, highlighting the importance of mycorrhizae for N and P uptake when both water and nutrients are limited. Since nutrient concentrations did not change, this also indicates that the absorbed nutrients were effectively used to sustain growth. This finding is particularly noteworthy, as water deficit typically leads to reduced growth as one of its earliest effects.

Carbon concentration (Figure 6C) and content (Figure 7C) remained stable across treatments, with the exception of a substantial variation between NM-LWR and M-NWR seedlings. This disparity is most likely due to the different physiological and allocation strategies used during drought stress and in response to ectomycorrhizal symbiosis. Non-mycorrhizal seedlings exposed to drought typically exhibit reduced stomatal conductance and photosynthesis, thereby limiting carbon assimilation (Dosskey et al., 1991; Davies et al., 1996). In response, biomass allocation often shifts toward root development to enhance water uptake, resulting in increased root: shoot ratios in non-mycorrhizal individuals (Davies et al., 1996). In contrast, mycorrhizal seedlings grown under ideal watering circumstances benefit from increased nutrient absorption and development (Koele and Hildebrand, 2011), but must allocate a significant amount of their photosynthates to sustain the fungal symbiont (Langley et al., 2006). The carbon drain caused by ectomycorrhizal colonisation is estimated to account for 10–20% of the host's photosynthetic production (Langley et al., 2006; Smith and Read, 2008).

The allocation of nutrients to shoots and roots also showed interesting patterns. In all treatments except NM-LWR, N, P, and C were preferentially allocated to the shoots, which aligns with the increased shoot biomass observed in these treatments. However, in NM-LWR seedlings, the higher root-to-shoot ratio suggests a greater allocation of resources to belowground parts. This could be a response to drought treatment, as the seedlings attempt to maximize water uptake through increased root growth and metabolic activity, even at

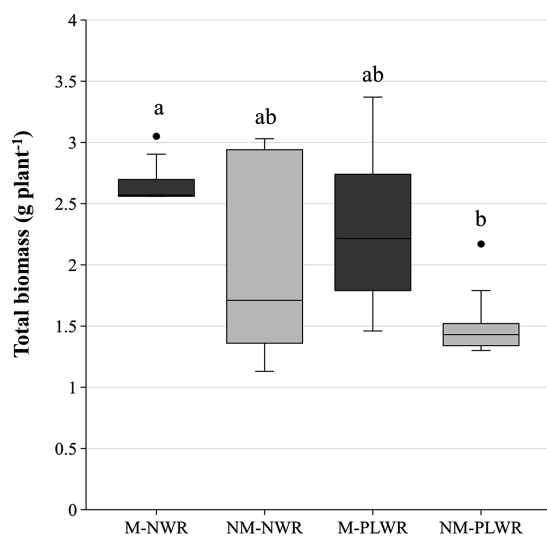


FIGURE 10

Exp 2: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on plant total biomass. The box plot displays the IQR of the scores, with the lower edge of the box indicating the first quartile and the upper edge marking the third quartile. The horizontal line inside each box represents the median score. The whiskers extend to the smallest and largest values within $1.5 \times$ IQR from the first and third quartiles. Scores outside this range are considered outliers and are depicted as individual points. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. PLWR: prolonged low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

the expense of shoot biomass. Such resource allocation strategies are commonly observed in plants under water-limited conditions, where prioritizing root growth can enhance access to water and improve drought tolerance. Carbon allocation, in particular, is generally prioritized to tissues increasing the uptake of limiting resources (Freschet et al., 2018).

4.1.2 Moderate and prolonged low water regimes

Under MWR conditions, we did not observe the same beneficial effects of EcM fungus inoculation as seen under LWR. One possible explanation is that MWR did not induce sufficient stress in *P. sylvestris*, which is known to be relatively drought-tolerant (Bose et al., 2020), to affect growth. Seedlings maintained stable water potential through stomatal regulation under MWR, avoiding growth limitation despite reduced photosynthesis. The absence of a reduction in biomass under water deficit in Experiment 2, unlike in Experiment 1, may suggest physiological acclimation. In this experiment, only the well-watered control plants (NWR) received regular nutrient supplementation, while the drought-treated plants (LWR, MWR, PLWR) did not, in order to simulate the reduction in nutrient availability typically associated with water deficit.

Under PLWR conditions, a significantly reduced level of root colonization was observed (from 94.3% under NWR to 63.8%), indicating that the mycelium of *P. involutus* was adversely affected by prolonged drought treatment, or that the symbiosis was downregulated by the plant when the cost–benefit balance was no longer favorable. This reduction in colonization likely decreased the ability of the EcM

fungus to support the photosynthetic rate and growth of the plants, as observed under LWR. These results are consistent with those of Wang et al. (2021), who found that EcM fungal colonization in *P. tabulaeformis* seedlings was lowest under severe drought conditions and highest under moderate drought. Kennedy and Peay (2007) showed that the benefits of ectomycorrhizal symbiosis between *P. muricata* and *Rhizopogon* species are highly dependent on soil moisture. Under moderate moisture, mycorrhizal plants outperformed non-mycorrhizal ones, but under severe drought, reduced fungal colonization led to the collapse of mutualistic benefits. This suggests that EcM fungi are themselves sensitive to the severity of drought and that their ability to confer drought tolerance to seedlings may depend on maintaining an adequate level of root colonization. Hagenbo et al. (2021) found that ectomycorrhizal mycelial production and turnover in *P. pinaster* and *P. sylvestris* forests were highly responsive to short-term variations in precipitation. Mycorrhizal biomass showed strong seasonal fluctuations, with increased growth during wetter periods and sharp declines during drought, indicating a close association between fungal activity and water availability. Gehring et al. (2020) showed that chronic drought significantly reduced ectomycorrhizal abundance and diversity in *P. edulis*. Similarly, Swaty et al. (2004) discovered that drought stress decreased ectomycorrhizal colonization and fungal community variability, with severely drought-affected areas having fewer active root tips and a different fungal community structure, but, interestingly, trees under moderate drought stress showed the largest EcM colonization, indicating a nonlinear response of the symbiosis to water availability. Inoculated seedlings subjected to PLWR that were earlier exposed to MWR and recovery phase showed no significant differences in leaf gas exchange parameters compared to the NWR controls, except for transpiration rate that was significantly lower (Figures 11A–D). On the other hand, NM-PLWR seedlings showed significantly lower transpiration rate and stomatal conductance compared to the seedlings in well-watered conditions, but not photosynthetic rate or water use efficiency. This finding suggests the possibility of drought-induced plant memory or a priming effect, wherein plants that have experienced a stress event “remember” it and adjust their responses to future stress (Bruce et al., 2007). Priming can be defined as the potential of plants to memorize environmental stress events and to strengthen their response to repeated stress (Harris et al., 2023).

During recovery, these stored stress imprints allow the plant to respond more quickly or effectively to subsequent stress events, thanks to the accumulation of metabolites and epigenetic modifications (Jacques et al., 2021). In our case, the seedlings’ ability to recover rapidly after PLWR suggests that some form of drought memory may be involved. However, more in-depth investigations, including genetic and molecular analyses, would be required to confirm whether such a priming effect is really present in *P. sylvestris*. Although some studies have demonstrated a priming effect to drought induced by EcM fungi in poplar trees (Luo et al., 2009) and pedunculate oak (Kebert et al., 2023), it is not possible to affirm the EcM fungus was involved in a possible priming effect in our study. Remarkably, Erlandson et al. (2022) demonstrated that long-term drought causes the EcM fungus *S. pungens* to exhibit a transcriptional acclimation response. Specifically, the fungus, showed no significant change in regulation of gene expression activity, when exposed to chronic drought. This finding suggests that some EcM fungi may undergo a molecular shift towards drought tolerance, potentially contributing to the host plant’s

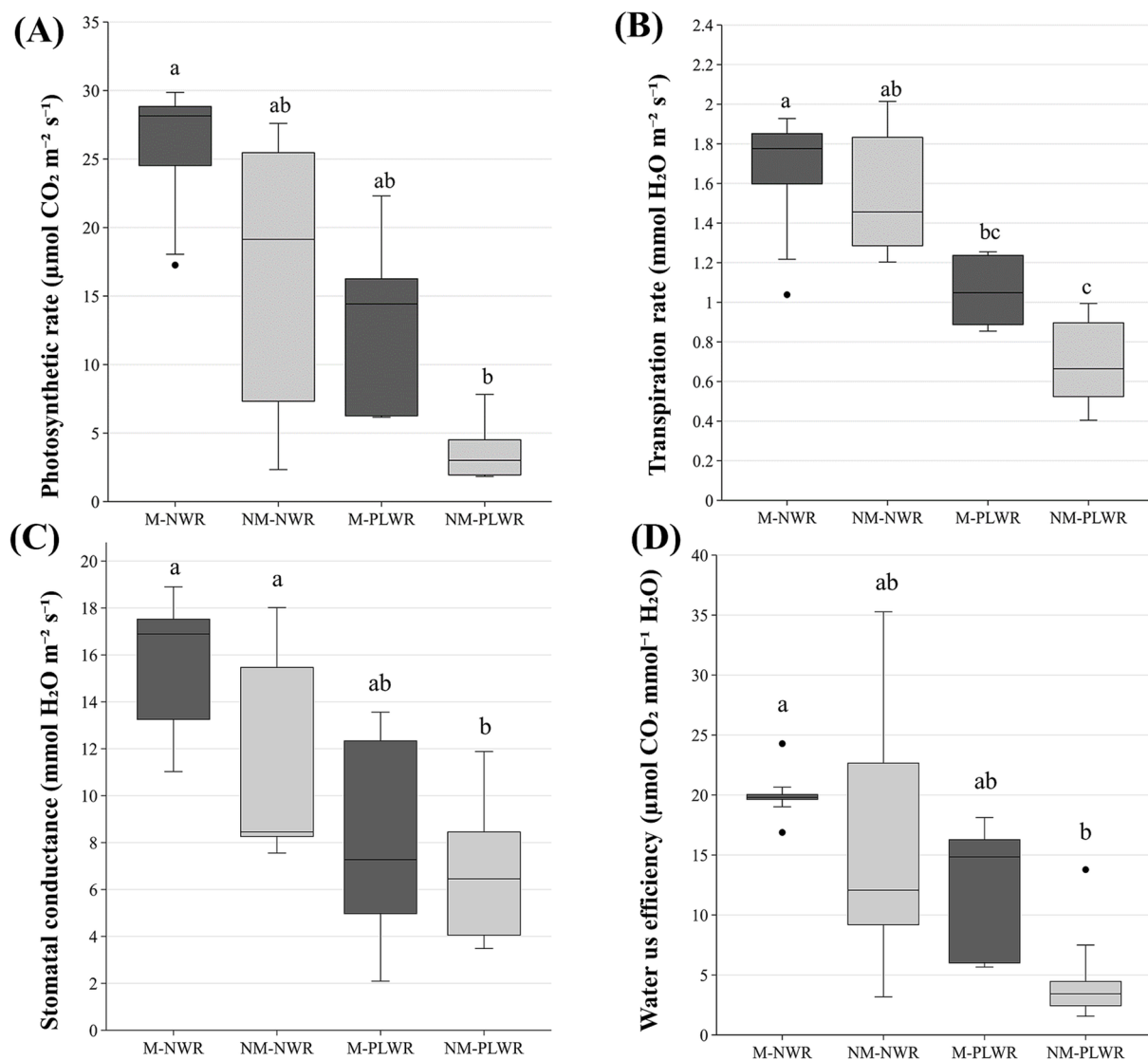


FIGURE 11

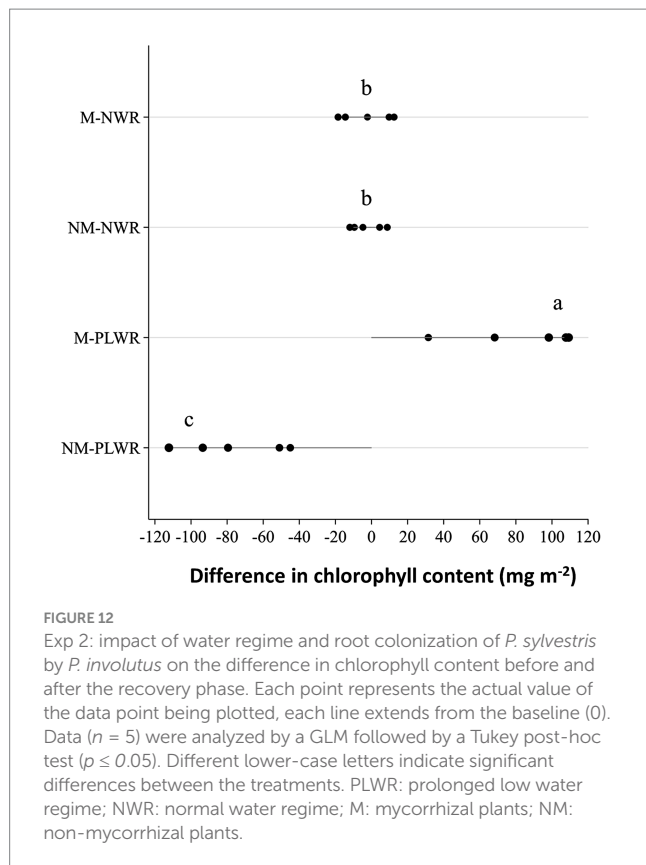
Exp 2: impact of water regime and root colonization of *P. sylvestris* by *P. involutus* on (A) photosynthetic rate, (B) transpiration rate, (C) stomatal conductance and (D) water use efficiency. The box plot displays the IQR of the scores, with the lower edge of the box indicating the first quartile and the upper edge marking the third quartile. The horizontal line inside each box represents the median score. The whiskers extend to the smallest and largest values within $1.5 \times \text{IQR}$ from the first and third quartiles. Scores outside this range are considered outliers and are depicted as individual points. Data ($n = 5$) were analyzed by a GLM followed by a Tukey post-hoc test ($p \leq 0.05$). Different lower-case letters indicate significant differences between the treatments. PLWR: prolonged low water regime; NWR: normal water regime; M: mycorrhizal plants; NM: non-mycorrhizal plants.

increased resilience. Although it is unknown if *P. involutus* exhibits comparable behavior, these fungal acclimation processes could significantly influence how the host plant responds to drought over time.

On the other hand, *P. involutus* root colonization was significantly reduced under PLWR conditions even after recovery phase, indicating that the EcM fungus was also strongly impacted by prolonged drought treatment. This decrease in *P. involutus* root colonisation is consistent with prior research demonstrating the harmful effects of drought on EcM fungi. For instance, Parke et al. (1983) found that mycorrhizal colonization in *Pseudotsuga menziesii* seedlings declined with water stress, with non-watered plants showing fewer surviving mycorrhizal root tips than well-watered controls. Coleman et al. (1989) also showed that the growth of EcM fungi, including *Paxillus* species, was

severely inhibited under low water conditions, indicating a direct susceptibility of fungal hyphae to water stress in pure culture. Supporting these controlled-environment results, Nickel et al. (2018) discovered that, in a temperate forest, 3 years of experimental summer drought resulted in a quantitative decrease in ectomycorrhizal colonization of fine roots, including in conifer species, while maintaining a relatively stable fungal community composition. Collectively, these investigations offer strong evidence that extended drought conditions impair both the colonization of roots and the development of the EcM fungi.

This reduction in colonization may have decreased the potential role of the EcM fungus in helping plants to recover from drought. Based on our data, it is difficult to definitively conclude whether the EcM fungus played a role in the potential priming effect observed in



the seedlings. However, the reduced colonization levels and the decreased physiological benefits under PLWR suggests that the mycorrhizal symbiosis may have been compromised under more extreme drought conditions.

4.2 Effect of *P. involutus* and water regime on seedlings' recovery

Regardless of the water regime applied, all seedlings, whether mycorrhized or not, exhibited similar levels of physiological parameters after 6 days. No significant differences were observed compared to the control groups under NWR. This suggests that the seedlings were able to re-establish adequate photosynthetic rates and WUE after the period of drought treatment. However, nutrient uptake during the recovery phase revealed that seedlings in the M-NWR treatment had a higher ability to take up NH_4^+ , suggesting that mycorrhizal colonization may facilitate more efficient nutrient uptake during the recovery phase (Figure 9), allowing them to maintain a higher photosynthetic rate compared to the other treatments (Figure 8), a phenomenon that is consistent with field observations of EcM mycelium production tracking moisture availability (Hagenbo et al., 2021; Querejeta et al., 2009). The functional rebound of EcM mycelium with improved moisture, as suggested by Hagenbo et al. (2021), could support both nutrient availability and possibly plant recovery. This is further corroborated by León-Sánchez et al. (2018), who showed in a 4-year manipulative study in a semi-arid shrubland that the decline in EcM presence, under rainfall reduction and

warming, was correlated with reductions in foliar nitrogen, phosphorus, and dry matter content, reflecting impaired nutrient status and declining plant tissue quality. Nonetheless, by the end of the recovery period, seedlings from all treatments had depleted the NH_4^+ present in the solution, indicating that even non-mycorrhized seedlings were able to resume normal nutrient uptake after drought treatment. These results imply that, while EcM fungi may confer an advantage during the initial stages of recovery, the plants themselves can restore their nutrient uptake capacity.

During the recovery phase, an increase in chlorophyll content was observed in the M seedlings, while a reduction in chlorophyll was seen in the NM seedlings that had been exposed to PLWR (Figure 12). This discrepancy could be due to the damage to Photosystem II or nutrient limitations in NM seedlings, particularly those in the PLWR treatment, which may have hindered the rapid production of chlorophyll when water became available again. In contrast, M seedlings appeared to recover more efficiently, likely due to the increased nutrient availability provided by the EcM fungal symbiosis. Yin et al. (2018) found that inoculation with *S. luteus* inhibited the reduction of chlorophyll content in *P. sylvestris* var. *mongolica* seedlings under drought conditions, highlighting the potential role of EcM fungi in preserving chlorophyll content and protecting the photosynthetic machinery during drought. Our findings suggest that a similar mechanism may be at play in the recovery of *P. sylvestris* seedlings inoculated with *P. involutus*, particularly under prolonged drought conditions.

In conclusion, the results of our study demonstrate that the EcM fungus *P. involutus* plays a crucial role in growth and physiology of *P. sylvestris* seedlings under drought-simulated conditions. Mycorrhized seedlings exhibited enhanced shoot biomass, improved photosynthetic rates, and increased nutrient uptake compared to non-mycorrhized seedlings, particularly under low water conditions. Under a moderate water regime, the stress was not sufficient to affect the growth of the plants and also the effect of the EcM fungus could not be evidenced. Also, the benefits of EcM symbiosis were no longer visible under prolonged low water conditions, likely due to a reduction in root colonization by the fungus. The potential for drought memory or priming effects in *P. sylvestris* seedlings warrants further investigation, particularly in relation to the role of EcM fungi in mediating these processes.

Overall, our findings highlight the importance of considering both plant and fungal responses to drought when assessing the resilience of forest ecosystems in the face of climate change. Field-based studies have shown that EcM fungi are highly sensitive to changes in water availability (Bell and Adams, 2004). For instance, Fernandez et al. (2023) demonstrated that climate-induced drought disrupts EcM interaction networks in boreal-temperate ecotones, leading to a breakdown in symbiotic connectivity that may compromise forest stability. Similarly, Hagenbo et al. (2021) reported that in Mediterranean forests dominated by *P. sylvestris*, *P. pinaster*, and *Quercus ilex*, the production and turnover of EcM mycelium were strongly influenced by drought intensity, with lower mycelial output observed under dry conditions. These field observations underscore that the functional capacity of both partners in the symbiosis can be impaired under climate stress, affecting nutrient dynamics, carbon exchange, and ultimately forest ecosystem resilience. Our findings reinforce the broader

understanding that the ability of EcM fungi to tolerate environmental stress—such as drought—plays a pivotal role not only in their own survival but also in shaping plant physiological responses. A recent review highlights the stress tolerance mechanisms of EcM fungi directly influence the outcome of the symbiosis, with cascading effects on forest ecosystem functioning and resilience (Branco et al., 2022). In the face of accelerating climate change, accounting for the dual responses of both host plants and their fungal symbionts is thus essential for predicting ecosystem stability and guiding conservation strategies.

It is also important to acknowledge that the semi-hydroponic cultivation system used in these experiments simplifies the complex interactions occurring in natural soil environments and may not fully replicate field conditions. However, our findings align well with those from several field studies, showing a positive role of EcM fungi in supporting plant nutrient uptake and physiological performance under water-limited conditions (e.g., Querejeta et al., 2009; Fernandez et al., 2023). They also document reduced ectomycorrhizal colonization and function under prolonged drought stress (e.g., Bell and Adams, 2004; León-Sánchez et al., 2018; Gehring et al., 2020; Hagenbo et al., 2021). These parallels suggest that, despite the controlled nature of our experimental system, the mechanisms we observed are ecologically relevant and reinforce broader patterns documented in natural ecosystems.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

PM: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. IL: Formal analysis, Investigation, Supervision, Validation, Writing – review & editing. RV: Data curation, Formal analysis, Writing – review & editing. JC: Data curation, Formal analysis, Writing – review & editing. QP: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. SD: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2025.1574237/full#supplementary-material>

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