Functional characterization of orchid mycorrhizal fungi isolated from *Anacamptis morio* in Northern Italy

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Research Article

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Abstract

Orchid mycorrhizal (ORM) fungi are essential for seed germination and plant development, yet their functional diversity remains poorly understood. In this study, we isolated and characterized rhizoctonia-like fungi from roots of the terrestrial generalist orchid *Anacamptis morio* collected across nine populations in Northern Italy. Sequencing and phylogenetic analyses of the ribosomal Internal Transcribed Spacer (ITS) identified isolates as belonging to the families Ceratobasidiaceae and Tulasnellaceae. A subset of isolates was functionally characterized for growth responses under different temperature regimes simulating current and future climate scenarios, for metabolic profiles using BIOLOG® carbon utilization assays and for the ability to induce symbiotic seed germination. Isolates exhibited marked functional variability across all tested traits. Growth responses to increasing temperature were strain-specific, suggesting differential resilience to climate change. Metabolic profiling revealed distinct substrate utilization strategies among isolates. Symbiotic performance also varied considerably, with some isolates promoting advanced protocorm and plantlet development and others showing limited or no germination-promoting capacity.

These findings demonstrate functional heterogeneity of ORM fungi associated with adult plants of *A. morio*, and suggest that a portfolio of different symbionts may contribute to orchid performance under different developmental and/or environmental conditions.

Introduction

Orchid biology is fascinating because these plants are highly successful exploiters of other organisms throughout all their life stages, from seed germination to flower pollination. Orchid seed germination and development in nature are entirely dependent on symbiotic associations with orchid mycorrhizal (ORM) fungi, and the sophisticated mechanisms to attract pollinators have likely contributed to make the Orchidaceae one of the largest families of flowering plants, comprising approximately 31,000 recognized species (POWO 2025). This plant family is also among the most globally threatened, with approximately 1,000 species (nearly 50% of the evaluated taxa) considered endangered according to IUCN Red List criteria (IUCN 2025).

Terrestrial orchids are particularly dependent on ORM fungi in the early life stages because their minute seeds, which are devoid of an endosperm and contain an immature embryo, develop into a post-embryonic structure (the protocorm) incapable of photosynthesis (Rasmussen 1995). Thus, seed germination and protocorm development require an external carbon source (Smith and Read 2008; Favre-Godal et al. 2020). ORM fungi colonize germinating seeds and protocorm cells, forming coiled hyphal structures known as pelotons. Being surrounded by the invaginated plant plasma membrane, pelotons are thought to be the site of nutrient transfer to the host plant (Kuga et al. 2014; Dearnaley et al. 2016). Adult orchids are also mycorrhizal in nature and the ORM fungi colonize the cortical cells of mature roots, forming pelotons whose function in nutrient transfer is supported by stable isotope (Cameron et al. 2006; Zahn et al. 2023) and transcriptomic studies (Fochi et al. 2017; Valadares et al. 2020, 2021).

Because of their pivotal role in plant growth, the presence of appropriate ORM fungal partners is critical for the establishment and persistence of orchid populations and communities (McCormick et al. 2018; Li et al. 2021). Expanding knowledge on the diversity and functional abilities of the mycorrhizal fungi associated with orchid roots is therefore essential to better understand their biology and to support effective conservation and restoration efforts.

ORM fungal taxa have been identified using culture-dependent (isolation) and culture-independent (i.e., metabarcoding) approaches, which have highlighted rhizoctonia-like fungi as the most common symbionts in photosynthetic orchids (Smith and Read 2008; Mennicken et al. 2024). Rhizoctonia-like fungi occur worldwide and belong to a polyphyletic group of basidiomycetes including Tulasnellaceae, Ceratobasidiaceae (order Cantharellales), and Serendipitaceae (Sebacinales) (Li et al. 2021). These fungi are generally considered soil saprotrophs capable of degrading complex organic matter through the release of extracellular enzymes like hydrolases, lyases, and oxidoreductases (Nurfadilah et al. 2013; Zhao et al. 2021). These enzymes can break down complex organic macromolecules (e.g., cellulose, lignin, starch) into smaller units that can be absorbed for nutrient acquisition and transfer to the plant (Smith 1967). Genes coding for these classes of enzymes have been found in the genome of representative isolates of these three fungal families (Kohler et al. 2015; Miyauchi et al. 2020). However, metabarcoding studies in distant geographic regions indicate a poor ability of some ORM fungi to grow outside the host in the surrounding soil (Voyron et al. 2017; Egidi et al. 2018; Kaur et al. 2019; Hartvig et al. 2024), thus raising questions on their actual saprotrophic abilities in nature. Thus, despite their ecological importance, the functional diversity of ORM fungi remains insufficiently explored.

Although metabarcoding is a powerful tool for characterizing the taxonomic diversity of ORM fungi in natural orchid populations, it does not provide pure fungal cultures, which are essential for functional characterization. Traits such as symbiotic effectiveness, responses to environmental conditions, and metabolic capabilities have therefore been investigated in only a limited number of cultured ORM fungal isolates (Zhao et al. 2021; Novotná et al. 2023).

In this study, we isolated a range of ORM fungi from roots of the terrestrial orchid *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase across multiple populations in Northern Italy. This generalist orchid species can host a diverse community of ORM fungi (Ercole et al. 2015a; Jacquemyn et al. 2015). We hypothesized that members of this ORM fungal community may differ in their physiological, metabolic or symbiotic abilities. We therefore (i) characterized their taxonomic diversity based on ITS sequence data, (ii) evaluated their growth responses to increasing temperature, iii) analyzed their metabolic profiles, and (iv) assessed their ability to promote symbiotic seed germination and protocorm development. By integrating functional

and phylogenetic approaches, this study provides new insight into the ecological specialization and adaptive potential of ORM fungi, highlighting their possible role in mediating orchid resilience under ongoing environmental and climate change.

Materials and Methods

Plant sampling and root treatment

Roots were collected in Spring 2022 from adult plants of *Anacamptis morio*. Five individuals from nine plant populations were sampled, and a complete description of the population features is reported in Table 1. Although all populations were located in Northern Italy, they represented two biogeographic regions (Alpine for SIG, and Continental for the others) and four habitats (urban park for BOL, low-altitude xeric grassland in the case of BMA, croplands in the case of ROC and PIA and montane meadow for SIG).

Individuals were randomly selected from the population and no additional individuals were dug out when the roots were few or small. Survival into the following growing season was observed for the majority of the sampled plants. This strategy was adopted to minimize our impact on the populations. Two to three roots per individual plant were collected in the field, depending on the root availability, and processed as soon as possible following the protocol described by (Calevo and Duffy 2023). Briefly, roots were rinsed under tap water to remove soil and debris, surface sterilized in a 1% sodium hypochlorite solution for three minutes and washed three times in sterile distilled water for 10 min before fungal isolation.

Table 1
Overview of the sampled orchid populations and associated fungal isolates, including site description, GenBank accession numbers of the best BLASTn results, fungal identification, and corresponding UNITE Species Hypotheses (SH). In bold the isolates selected for further analyses, later referred to as AM7, AM12, AM34, AM40, AM44 and AM45.

Population ID	Population site	Population description	Fungal isolate ID	Closest match in GenBank				Organism	SH (UNITE DB)
				Accession number	Query cov.	E value	Percent identity		
MCE	Monte Cecilia	Population hosted in a vineyard in NE-Italy	AM1.1D	KF537646	99%	1.00E-111	90.68%	*Tulasnella* sp.	SH0984058.10FU
			AM1.2C	KF537646	100%	8.00E-168	98.27%	*Tulasnella* sp.	SH0984058.10FU
			AM1.10D	MN545109	89%	1.00E-56	81.69%	Tulasnellaceae *sp.*	SH0984058.10FU
			AM3.4C	KF537646	99%	1.00E-170	98.02%	*Tulasnella* sp.	SH0984058.10FU
			AM4.1C	KF537646	100%	2.00E-168	97.21%	*Tulasnella* sp.	SH0984058.10FU
			AM4.3A	KF537646	100%	0.00E + 00	99.46%	*Tulasnella* sp.	SH0984058.10FU
			AM4.3B	KF537646	99%	2.00E-158	95.34%	*Tulasnella* sp.	SH0984058.10FU
			AM4.5E	OR507100	97%	6.00E-55	81.53%	Tulasnellaceae *sp.*	SH0984058.10FU
BOL	Bologna	Population growing in a public garden in the sub-urban area of the city of Bologna	**AM7.1C**	OM971071	100%	0.00E + 00	99.78%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.2A	OM971071	100%	0.00E + 00	99.26%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.2B	OM971071	100%	0.00E + 00	99.78%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.2D	OM971073	100%	0.00E + 00	98.92%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.3C	OM971071	100%	0.00E + 00	98.70%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.4B	OM971071	100%	0.00E + 00	96.85%	Ceratobasidiaceae sp.	SH0961459.10FU
			AM7.4C	OM971071	100%	0.00E + 00	97.28%	Ceratobasidiaceae sp.	SH0961459.10FU
BMA	Bosco Marengo	Wide population in a riparial xeric grassland alongside the Po River	AM12.2C	ON920828	99%	0.00E + 00	95.42%	*Tulasnella* sp.	SH0986850.10FU
			AM12.2D	ON920828	100%	1.00E-155	97.58%	*Tulasnella* sp.	SH0986850.10FU
MOG	Monte Ogheri	NE-Italy	AM21.1B	OR507124	100%	0.00E + 00	99.55%	Tulasnellaceae sp.	SH0986850.10FU
PIA	Pianlago	Wide and dense population growing on the edge of an "organic wheat" field on the N-Apennines	AM34.2A	OR507121	99%	0.00E + 00	98.70%	Tulasnellaceae sp.	SH0984058.10FU
			AM34.2B	OM976766	100%	3.00E-138	90.86%	Tulasnellaceae sp.	SH0984058.10FU
			AM34.2D	OR507121	99%	4.00E-103	84.91%	Tulasnellaceae sp.	SH0984058.10FU
			AM34.3B	OR507121	100%	0.00E + 00	97.42%	Tulasnellaceae sp.	SH0984058.10FU
			AM34.4D	OR507121	100%	0.00E + 00	96.99%	Tulasnellaceae sp.	SH0984058.10FU
ROC	Roccabianca	Vineyard population in N-Apennines	AM40.3C	MN947298	100%	2.00E-104	92.50%	*Tulasnella* sp.	SH0984088.10FU
			AM40.3D	MN947299	100%	0.00E + 00	99.20%	Tulasnellaceae sp.	SH0984088.10FU

Population ID	Population site	Population description	Fungal isolate ID	Closest match in GenBank				Organism	SH (UNITE DB)
				Accession number	Query cov.	E value	Percent identity		
SIG	Sigliodo	Small population growing in montane meadow (W-Alps)	**AM44.2D**	MT506704	100%	2.00E-123	97.74%	*Tulasnella* sp.	SH0984106.10FU
			AM45.2A	JF926503	100%	0.00E + 00	96.77%	*Tulasnella* sp.	SH0984083.10FU
			AM45.3B	JF926503	100%	2.00E-76	98.24%	*Tulasnella* sp.	SH0984083.10FU
			AM45.4C	JF926503	100%	4.00E-105	93.33%	*Tulasnella* sp.	SH0984083.10FU
BMO	Bric Montariolo	Disturbed sub-mediterranean shrubland	AM49.1D	ON920828	99%	1.00E-100	90.34%	*Tulasnella* sp.	SH0986850.10FU
SVI	San Vito	Private garden in the Monferrato hills region	AM51.2A	OR507124	100%	0.00E + 00	98.74%	Tulasnellaceae sp.	SH0986850.10FU
			AM54.2B	ON920828	100%	0.00E + 00	99.60%	*Tulasnella* sp.	SH0986850.10FU
			AM54.2D	OR507124	100%	0.00E + 00	99.58%	Tulasnellaceae sp.	SH0986850.10FU
			AM54.3	OR507124	100%	0.00E + 00	99.76%	Tulasnellaceae sp.	SH0986850.10FU
			AM54.3A	OR507124	100%	0.00E + 00	97.34%	Tulasnellaceae sp.	SH0986850.10FU
			AM54.4C	OR507124	100%	0.00E + 00	97.29%	Tulasnellaceae sp.	SH0986850.10FU
			AM54.4E	OR507124	100%	1.00E-175	100.00%	Tulasnellaceae sp.	SH0986850.10FU
			AM55.2A	OR507124	100%	0.00E + 00	99.56%	Tulasnellaceae sp.	SH0986850.10FU

Fungal isolation

Potential ORM fungi were isolated from surface sterilized roots obtained from each plant population (448 root fragments in total). Surface sterilized roots were sectioned into small fragments under sterile conditions and fragments were plated on Petri dishes containing half strength potato dextrose agar (VWR, USA) supplemented with 5 ml/l streptomycin to inhibit bacterial growth. Petri dishes were incubated in complete darkness at 25°C and checked regularly for fungal growth. As soon as hyphae emerged from the root fragments, they were sub-cultured onto fresh medium to obtain pure fungal isolates. Fungal isolates that, despite the use of antibiotics, showed bacterial contamination were discarded. A total of 51 isolates were retained after morphological screening of features characteristic of rhizoctonia-like ORM fungi, namely colony morphology, right angle hyphal branching with a constriction near branching point, size and shape of monilioid cells. Isolates were stored on PDA medium at 4°C.

Identification and phylogeny of fungal isolates

For molecular identification, fungal isolates were grown in flasks containing malt extract broth (MEB, 2%) and mycelia were collected by filtration using a Buchner funnel after 5–7 days of culture at 25°C in the dark on an orbital shaker. The collected mycelium was lyophilized and mechanically ruptured in a beads beater (Retsch Gmbh MM 301, Germany). Total DNA was extracted with a modified CTAB method according to Schenk et al. (2023). The nuclear ribosomal ITS region was amplified with the ITS1-ITS4 primers (White et al. 1990) and the amplicons were sent to BioFab Research s.r.l. (Roma, Italy) for Sanger sequencing. Taxonomic sequence identification was performed using two complementary approaches: (i) sequences were compared to the NCBI nucleotide database using the BLASTn algorithm (Altschul et al. 1990) to retrieve closest matches, and (ii) sequences were assigned to Species Hypotheses (SH) by comparison with the UNITE database using a 1.5% sequence dissimilarity threshold. To better understand the relationships between the *A. morio* isolates and previously published representative sequences of ORM fungi, phylogenies were constructed separately for the Ceratobasidiaceae and Tulasnellaceae. ITS sequences were aligned with the nearest identified taxa revealed by BLAST searches as well as other reference sequences retrieved from GeneBank. Sequences were aligned using MUSCLE (Edgar 2004), and poorly aligned regions were removed using Gblocks. Maximum Likelihood (ML) phylogenetic analyses were conducted with RAxML (version 8.2.12, Stamatakis 2014) implemented in raxmlGUI 2.0 (version 2.0.10; Edler et al. 2021) using the GTR + G substitution model and rapid bootstrap with the autoMR criterion to assess branch support. *Multiclavula vernalis* (MVU66439) was used as an outgroup for the Tulasnellaceae and *Thanatephorus ochraceus* (EU218892) for the Ceratobasidiaceae.

Fungal growth rate at different temperatures

Mycelial growth rate was measured under three different temperatures representing observed historical records and projected future conditions according to the worst-case Shared Socioeconomic Pathways scenario (SSP585). Temperatures were selected based on the mean temperature of the warmest quarter (Bio10) under the high-emissions SSP585 scenario, using WorldClim v2.1 downscaled CMIP6 climate projections. For our study area, Bio10 values were 21.4°C for the historical period (1970–2010), 25.5°C for the mid-century projection (2041–2060), and 27.7°C for the late-century projection (2061–2080). For each isolate, a 6 mm diameter plug was collected from the actively growing edge of a fungal colony on MEA medium (2% malt extract, 2% agar) and placed in the centre of a Petri dish containing the same medium. Standard 9 cm Petri dishes were used for isolates AM12 and AM34, whereas 15 cm Petri dishes were used for isolates AM7, AM40, AM44, and AM45, which exhibited faster radial growth and reached the edges of standard plates within 14 days. The use of larger plates ensured that linear growth could be measured over the full 21-day incubation period without spatial constraints. Five biological replicates of each isolate were prepared. All cultures were incubated in the dark at the three temperature conditions. Petri dishes were scanned every 7 days up to 21 days using an Epson Perfection 2450 Photo scanner to quantify colony diameter. For each biological replicate, three independent measurements of colony diameter were recorded using Image J v 1.54g (Schneider et al. 2012), and their mean value was used in subsequent analyses. A generalized linear model (GLM) was first used to test the effects of isolate, temperature and replicate on colony growth. Since the replicate factor was not significant, it was excluded from the final model. The effects of isolate and temperature on growth were then assessed using a GLM with Gaussian errors. A non-parametric Kruskal–Wallis tests followed by Dunn's post-hoc comparisons with Bonferroni correction were applied to compare colony growth among isolates at each temperature. To analyze growth rate (cm day^{-1}), a beta regression model including isolate, temperature, and their interaction was fitted. Model assumptions were checked through residual diagnostics, and coefficients were exponentiated for interpretability. Graphical outputs and model visualizations were produced with ggplot2 and sjPlot packages in R.

Metabolic fingerprinting of fungal isolates by Phenotype MicroArray

Metabolic fingerprinting of the fungal isolates was performed using BIOLOG® FF MicroPlates™ (Biolog Inc., Hayward, CA, USA), containing 95 different carbon sources and a tetrazolium redox dye that is reduced during fungal metabolism, producing a purple color proportional to substrate utilization. Liquid cultures of each isolate were prepared in MEB 0.2% and incubated at 25°C for 1–2 weeks. Cultures were filtered under sterile conditions through a Büchner funnel and washed with sterile distilled water. Mycelium was then disrupted to obtain an homogeneous suspension. Inoculating fluid (0.05% water-agar) was added to an FF-IF tube to a final volume of 15 mL and the transmittance was adjusted to 100%. Mycelial suspension was added to the tube until transmittance reached 75% and 100 µL of the resulting solution was inoculated into each well of the BIOLOG® FF MicroPlates™. To minimize evaporation during the incubation period, sterile distilled water was added to the empty wells of the plates. Two technical replicates were prepared for each isolate and incubated at 25°C for 10 days. Absorbance at 490 nm was recorded every 15 minutes using the automated plate reader Odin L (Biolog Inc., Hayward, CA, USA). For each well, absorbance values of the negative control were subtracted to normalize for background signal, and negative values were set to zero. The area under the curve (AUC) was calculated and the AUC values of the two technical replicates were averaged to obtain a mean value for each isolate used for visualization. Principal component analysis (PCA) was performed to evaluate patterns in metabolic profiles. Heatmaps were generated with hierarchical clustering of both isolates and substrates using Euclidean distances and complete linkage. AUC data were scaled as z-scores either by column (to compare isolates across the same substrate) or by row (to compare substrate usage within each isolate). Heatmaps were generated in R (v4.2.1) using the pheatmap package (v 1.0.13).

Induction of orchid seed germination and protocorm development

The ability of fungal isolates to induce orchid seed germination and protocorm development was assessed using a single accession of seeds of *Serapias vomeracea* (Burm. f.) Briq. collected in Naples, Italy, nine months before the experiment. This species was used because germination assay with the available batch of seeds of *A. morio* was unsuccessful due to low seed viability. The use of fungal isolates obtained from *A. morio* to assess symbiotic seed germination and protocorm development in *Serapias vomeracea* is supported by the fact that *Anacamptis morio* and *S. vomeracea* frequently co-occur in Mediterranean grasslands and other open habitats, and often share overlapping pools of orchid mycorrhizal (ORM) fungi (Jacquemyn et al., 2015). Consistent with this pattern, four of the six fungal isolates used in this study (SH0986850.10FU, SH0984058.10FU, SH0984088.10FU, and SH0984083.10FU) have previously been reported as mycorrhizal associates of *S. vomeracea* (Girlanda et al., 2011). A sample from the seed batch was checked for viability using fluorescein diacetate (FDA) staining (0.5% w/v), and seed viability was estimated at 72%. Seeds were surface sterilized using a 1% sodium hypochlorite solution for 18 min, then rinsed three times in sterile distilled water for 5 min each. Seeds were sown on sterile oat agar (OA, 0.4% oat, 1% agar) previously inoculated with each fungal isolate and kept at 21°C in darkness, as described in (Ercole et al. 2015b). Seed germination and protocorm development were evaluated after three weeks on twelve biological replicates per isolate, and monitoring of plant development was continued up to four months. A score of germination ability was assigned to each isolate based on the stage of protocorm development, according to the embryos developmental stages suggested by Swarts and Dixon (2017). In addition, the percentage of germinated seeds (from stage 3) was calculated. For each isolate, a corrected germination rate (% germinated seeds relative to the viable seed fraction) was calculated. Differences among isolates were analysed using a Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction. Furthermore, the proportion of germinated seeds reaching each developmental stage (2–5) was calculated for each isolate to assess protocorm developmental progression. Protocorm development and plantlet formation was further monitored for up to six months.

Results

Fungal isolates from A. morio

About 51 fungal colonies were isolated from the roots of *A. morio* collected across nine sites. After amplification and sequencing of the ITS region, isolates with low quality sequences were removed and 38 isolates were retained for further analyses (Table 1). BLASTn searches of the NCBI nucleotide database and assignment to Species Hypotheses (SH) on the UNITE database revealed that isolates could be classified within Ceratobasidiaceae or Tulasnellaceae. None of the isolates could be assigned to Serendipitaceae. The phylogenetic trees (Supplementary **Fig. S1** and **Fig. S2**) showed that all isolates belonging to the family Ceratobasidiaceae clustered within a single well-supported clade in the genus *Ceratobasidium* (**Fig. S1**). By contrast, isolates identified in the family Tulasnellaceae clustered in separate clades inside the *Tulasnella* genus, indicating substantial diversity among isolates (**Fig. S2**). Representative isolates were selected from each major clade, based on their association with distinct reference lineages and SH clusters. Six fungal isolates in total (in bold in Table 1), representing the phylogenetic diversity observed in the trees, were then characterized for their ability to grow at different temperatures and on different substrates, and to induce seed germination. The colony morphology of these isolates on MEA medium is shown in Fig. 1.

Mycelial growth at different temperatures

Growth of the six fungal isolates was measured at three different temperatures to investigate the ability of these fungi to face increasing temperatures. Fungal colony growth was significantly affected by both factors “isolate” and “temperature”, whereas “biological replicate” did not have a significant effect and was therefore excluded from further analyses. At all temperatures, Kruskal–Wallis tests revealed significant differences among isolates (all $p < 0.001$). Overall, AM7 consistently exhibited the fastest growth across all temperatures, whereas AM12 was generally the slowest-growing isolate. Figure 2A shows that growth rate responses to increasing temperature varied among fungal isolates. Three isolates (AM40, AM44, AM45) exhibited an increase in colony diameter with increasing temperature, indicating enhanced growth at higher temperatures. Growth of AM12 and AM34 was not affected by temperature, while AM7 showed a reduction in growth at the higher temperatures. These trends were supported by the statistical model in Fig. 2B. Temperature alone had a significant overall effect on growth rate (estimate = 0.97, $p = 0.008$) relative to the reference strain AM12. Significant isolate × temperature interactions were observed for AM40, AM44 and AM45 (all $p < 0.001$), confirming that these isolates responded positively to increasing temperature. No significant interaction was detected for AM34 ($p = 0.243$); while the interaction between temperature and AM7 was negative and significant ($p = 0.006$), confirming a decrease in growth rate at higher temperatures.

Carbon usage by fungal isolates by Phenotype MicroArray

BIOLOG® FF Microplate™ were used to assess the ability of the six fungal isolates to utilize different substrates as the sole carbon source. The PCA in Fig. 3 shows that replicates from the same isolate generally clustered closely, with the exception of AM40. Moreover, isolates were clearly separated along both axes, showing differences in carbon utilization.

The heatmap in Fig. 4 illustrates the metabolic profiles, with each column representing a carbon source and each row an isolate. Data was scaled by column, thus enabling relative comparisons of substrate utilization among fungal isolates. The list of carbon sources, from left to right, is available in **Table S1**. Hierarchical clustering based on Euclidean distance revealed distinct patterns: AM7 and AM45 clustered together, showing broad utilization of substrates such as plant-derived carbohydrates (particularly simple sugars, disaccharides, and polyols); AM34, AM40, and AM44 displayed more heterogeneous patterns, with overall lower activity compared to the other isolates. AM12 was uniquely characterized by the utilization of amino sugars, amines, amides, polyols, and nucleotide-related substrates, suggesting a specialization toward nitrogen-rich resource pools.

The heatmap in **Fig. S3** also illustrates the metabolic profiles of the different isolates, but data was scaled by rows, thus allowing visualization of the substrates that were most actively metabolized by each specific isolate, rather than comparisons across isolates. The list of carbon sources, from left to right, is available in **Table S2**. Hierarchical clustering based on Euclidean distance revealed that the four *Tulasnella* spp. isolates AM34, AM40, AM44 and AM45 clustered together and shared a consistent substrate-use profile dominated by mono- and oligosaccharides, storage and polymer-derived carbohydrates, and cellulose/hemicellulose breakdown products, indicating a generalist metabolism centered on plant-derived carbon sources. In addition, isolates AM40 and AM45 showed preferential use of D-ribose, pointing to an extended ability to exploit pentose sugars potentially derived from nucleotide turnover. The *Ceratobasidium* sp. AM7 isolate showed an overall similar profile but clustered separately because of the ability to grow also on galactose-containing carbohydrates, including free D-galactose and galactosides such as lactose, lactulose, and melibiose (**Fig. S3**). Isolate AM12 showed again a distinct pattern in the use of substrates, characterized by preferential utilization of amino sugars (glucosamine and N-acetylated derivatives), organic nitrogen compounds (amines, amides, and nucleotide-related substrates), and a range of modified carbohydrates and polyols. While AM12 retained the ability to metabolize common plant sugars, its profile was notably enriched in nitrogen-containing and non-canonical substrates, distinguishing it from other isolates that primarily targeted plant-derived carbohydrate pools.

Induction of seed germination and protocorm development by the fungal isolates

The six fungal isolates showed different abilities to promote seed germination and protocorm development (Kruskal-Wallis p-value = 9.108e-10) of *S. vomeracea*, as shown respectively in Fig. 5A and 5B. Seeds co-cultured with isolate AM7 (*Ceratobasidium* sp.) remained imbibed but did not germinate, as well as most seeds inoculated with isolate AM44 (*Tulasnella* sp. SH0984106.10FU). Isolate AM45 (*Tulasnella* sp. SH0984083.10FU) promoted

development only up to stage 4, whereas inoculation with AM12 (*Tulasnella* sp. SH0986850.10FU), AM34 (*Tulasnella* sp. SH0984058.10FU) and AM40 (*Tulasnella* sp. SH0984088.10FU) resulted in a majority of symbiotic protocorms reaching stage 5 after four months.

After six months of co-culture, there were marked differences in plant size (Fig. 6): plantlets germinated in the presence of AM34 were considerably more developed than those obtained with the isolates AM40 and AM12, although all isolates induced protocorm development to stage 5 and leaflet formation. Mycorrhizal fungal structures (pelotons) were observed in the roots of plantlets inoculated with AM34 and AM40, whereas no pelotons were identified in the roots of plantlets inoculated with AM12 probably due to the tiny size of the plantlets (data not shown).

Discussion

This study highlights the remarkable functional diversity of orchid mycorrhizal fungi associated with *Anacamptis morio*, a generalist orchid in terms of mycorrhizal association (Bailarote et al. 2012; Ercole et al. 2015a), and plant ecology (Geppert et al. 2020). Although all isolates were obtained from roots of adult plants belonging to the same orchid species, they differed substantially in phylogenetic placement, thermal response, carbon utilization profiles, and symbiotic performance during seed germination and subsequent plant development. These findings demonstrate that co-occurring ORM fungi are not functionally equivalent and may differ in their capacity to support orchid development.

Phylogenetic analyses placed the isolates in distinct clades within the Tulasnellaceae and Ceratobasidiaceae, yet functional traits were not consistently associated with taxonomic relationships. Notably, the *Ceratobasidium* isolate did not appear functionally more divergent than the *Tulasnella* isolates, which exhibited marked variability despite their close taxonomic relatedness. These results suggest that functional traits in ORM fungi may vary considerably even among closely related taxa, highlighting the complexity of linking ecological functions to phylogenetic identity in orchid-associated fungi. Similar patterns of functional heterogeneity within phylogenetically related fungi have been reported in previous studies about ORM fungi (Jacquemyn et al. 2011; Calevo and Duffy 2023), and arbuscular mycorrhizal fungi (Van Der Heijden et al. 2004).

The distinct substrate-use profile of isolate AM12, which preferentially utilized nitrogen-rich compounds and amino sugars, contrasted strongly with the carbohydrate-oriented metabolism of the other isolates, which was observed also in previous experiments on ORM fungi (Adamo et al. 2020). The *Ceratobasidium* isolate AM7 exhibited a broader capacity to metabolize galactose-containing compounds than the other isolates. Similarly, isolates differed markedly in their growth responses under increasing temperature regimes, with some showing enhanced growth at higher temperatures and others being negatively affected (Fig. 2). The coexistence of multiple fungal taxa within the same habitat is likely facilitated by the divergence of generalist and specialist metabolic strategies, aligning with the classical specialist-generalist niche theory framework (Bebber and Chaloner 2022).

The present dataset does not allow us to determine whether the observed functional differences represent stable lineage-specific ecological traits or variability among isolates within the same lineage, as only a single representative isolate was functionally characterized for most clades. Previous studies have shown that ORM fungi can exhibit substantial intraspecific variation in physiological and symbiotic traits, including enzymatic activity, environmental tolerance, and effectiveness in supporting orchid development (Martos et al. 2012; Adamo et al. 2020; Calevo and Duffy 2023). Further studies including multiple isolates within the same phylogenetic groups will therefore be necessary to evaluate the extent to which functional traits are conserved across ORM fungal lineages.

The germination assays further demonstrated that fungal isolates differed in their ability to support seed germination and protocorm development. Whereas some isolates (AM34 and AM40) promoted plantlet formation, others induced only limited plantlet growth (AM12) or protocorm development (AM45 and AM44), or failed entirely to support seed germination (AM7). Such variability is consistent with the highly dynamic and context-dependent nature of orchid–fungus interactions (Rasmussen 1995; Dearnaley et al. 2016). The fungal isolates used in this study were obtained from roots of adult *A. morio* plants but tested on seeds of *S. vomeracea* because of the low viability of our batch of *A. morio* seeds. Both orchid species are considered generalist and frequently share overlapping pools of ORM fungi (Jacquemyn et al. 2015). Notably, *A. morio* and *S. vomeracea* co-occur in close spatial proximity in some of the sampled areas of this study, suggesting that at least part of the ORM fungal community associated with *A. morio* may also be available to *S. vomeracea* under natural conditions. The contrasting abilities of the fungal isolates to induce seed germination and early plant development may indicate that the same fungal taxa may establish functionally different associations depending on the orchid host species, and possible host-specific responses should be further investigated. An alternative, non-mutually exclusive explanation, is that the isolates recovered from adult roots may play different ecological roles during the orchid life cycle. Increasing evidence indicates that ORM fungal communities can shift across developmental stages, with germinating seeds, protocorms, and adult plants associating with partially overlapping but functionally distinct fungal partners (McCormick et al. 2004; Bidartondo et al. 2004; Wang et al. 2021). Some fungi may be particularly effective during early protocorm development, when carbon transfer from the fungus to the achlorophyllous embryo is essential, whereas others may primarily function in mature photosynthetic plants (Bidartondo and Read 2008; Ventre Lespiaucq et al. 2021).

The observed differences in fungal growth responses to increasing temperature may also have ecological implications for orchid persistence under climate change. Studies based either on historical series (Geppert et al. 2020; Goury et al. 2025), or on predictive inference (Charitonidou et al. 2022; Nepote Valentin et al. 2025) are observing a general shift in orchids distribution, but the overlooked factor of these studies is that orchid recruitment in natural populations strongly depends on the local availability of compatible fungal symbionts (McCormick et al. 2018; Li et al. 2021). Thus, changes in temperature may alter both fungal community composition and symbiotic performance. Isolates such as AM40, AM44, and AM45 exhibited enhanced growth at elevated temperatures, suggesting potential resilience under warming scenarios, whereas others showed reduced growth. Because orchid

establishment relies on successful seed germination and protocorm development mediated by ORM fungi, differential thermal sensitivity among fungal partners could ultimately influence orchid distribution, recruitment success, and population stability under future climatic conditions.

Conclusions

Based on our findings, we may hypothesize that the functional diversity of ORM fungi constitutes a symbiotic portfolio for the host, in which complementary fungal partners may differentially support orchid recruitment, development, and resilience, thereby buffering populations against environmental change. Our results also highlight the importance of considering functional variation among fungal isolates when addressing orchid ecology, conservation, and restoration strategies.

Declarations

Data Accessibility Statement

The ITS sequences of the ORM fungal isolates of this study are available in GenBank under the accession numbers: XX$$$$$$$ - XX$$$$$$$.

Conflicts of Interest

Authors have no competing interests to declare.

Fundings

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Author Contribution

MF performed most of the experimental work, analyses and figures. MA co-designed the study, contributed substantially to statistical analyses and interpretation, and supervised the work. ASF provided methodological support and supervision, particularly for laboratory work. SP drafted the manuscript and supervised the work. MG and SP jointly conceived and supervised the project and secured funding. All authors contributed to the interpretation of the results, critically revised the manuscript, and approved the final version.

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Data Availability

The ITS sequences of the ORM fungal isolates of this study are available in GenBank under the accession numbers: XXXXX - XXXXX.

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Figures

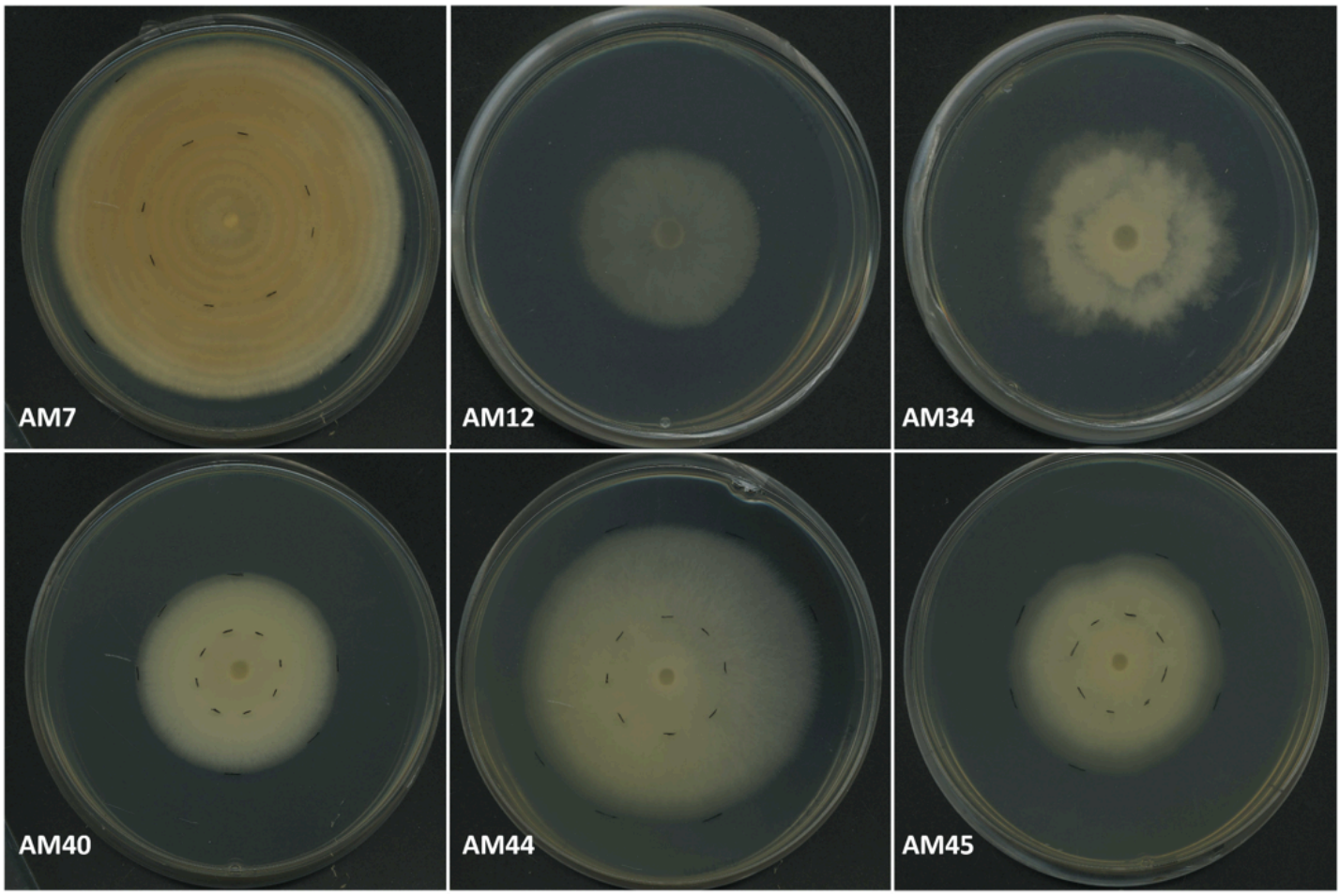

Figure 1

Colony morphology on MEA medium 14 days after inoculation. Colony morphology of the fungal isolates selected for further characterization. AM7 represents the single clade of *Ceratobasidium* sp., whereas all other isolates were identified as *Tulasnella* spp. Dashed lines were used for measurement purposes and indicate colony growth after 7 days.

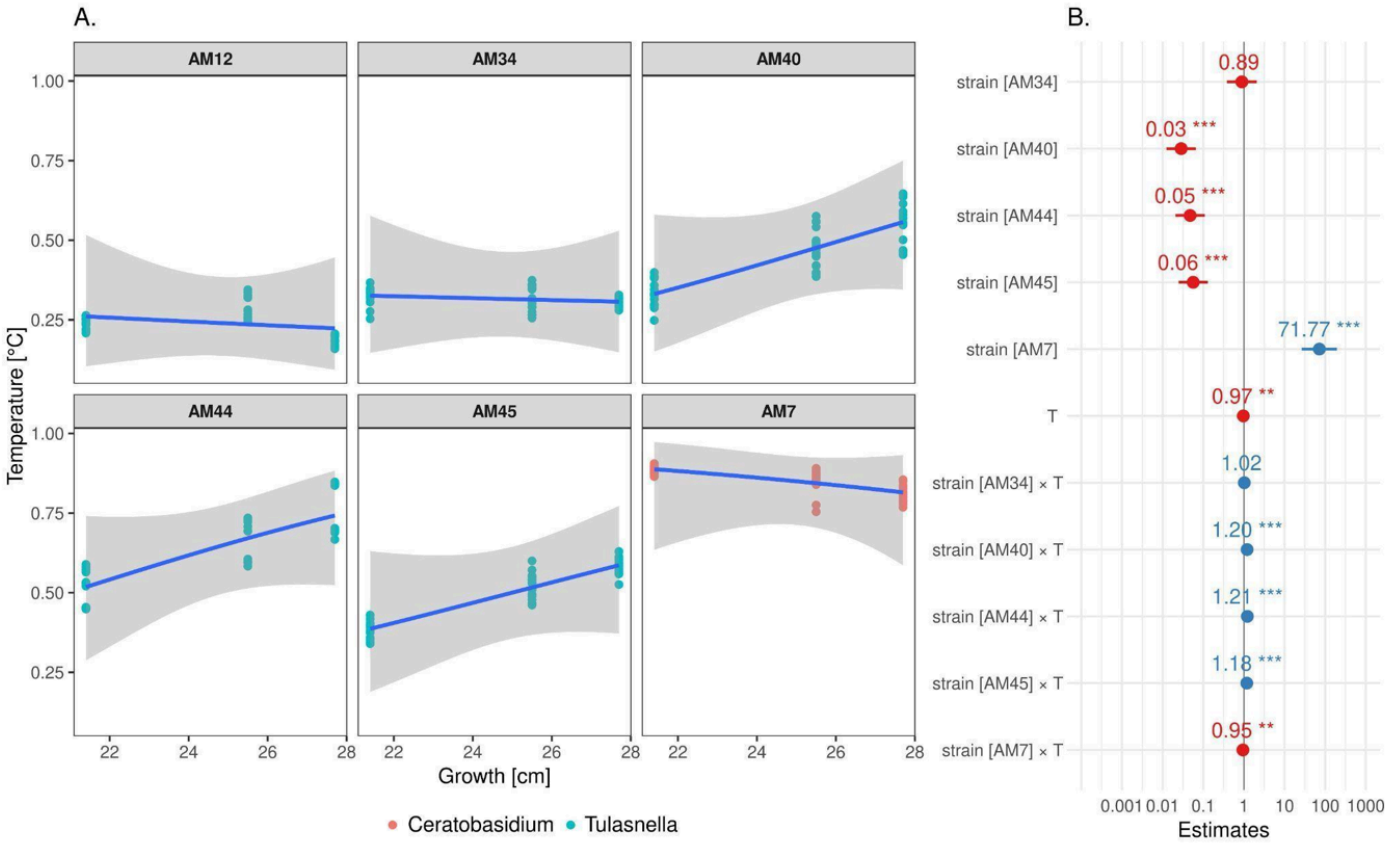

Figure 2

Influence of fungal isolate and temperature on fungal growth. Influence of fungal isolate and temperature on fungal growth was estimated using a generalized linear model (GLM). A. Relationship between fungal growth (cm) and temperature (°C) for each isolate. Points represent observed values, colored by fungal genus (*Ceratobasidium* and *Tulasnella*). Blue lines show GLM-fitted trends, and grey bands indicate 95% confidence intervals. B. Model coefficient estimates for strain, temperature (T), and isolate × temperature interaction terms, plotted relative to the reference isolate AM12. Points and horizontal bars show estimates and confidence intervals, respectively; values greater or lower than 1 indicate positive or negative effects on growth, respectively. Asterisks indicate statistical significance (** for p-values < 0.01, *** for p-values < 0.001).

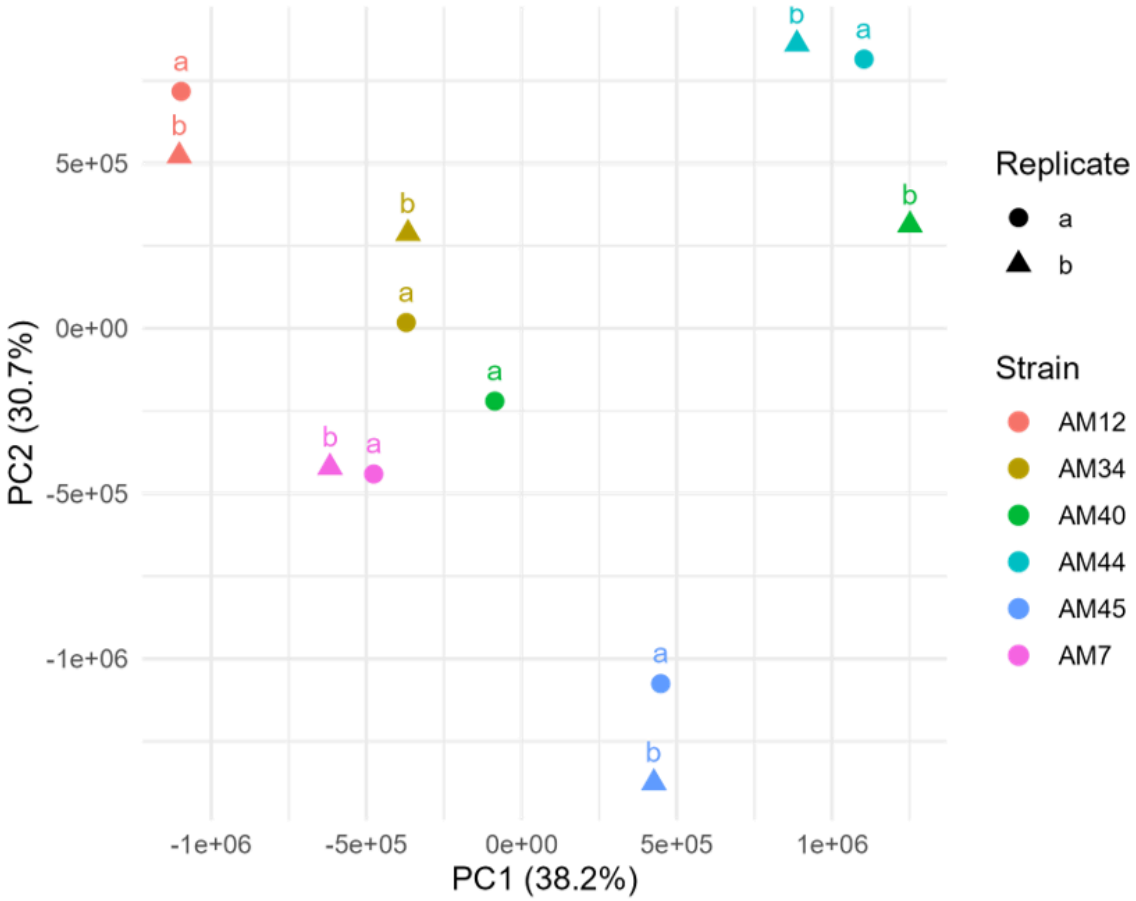

Figure 3

Principal component analysis of area under the curve (AUC) values. Each point represents a biological replicate, and the distance between points reflects dissimilarity in carbon source utilization profiles.

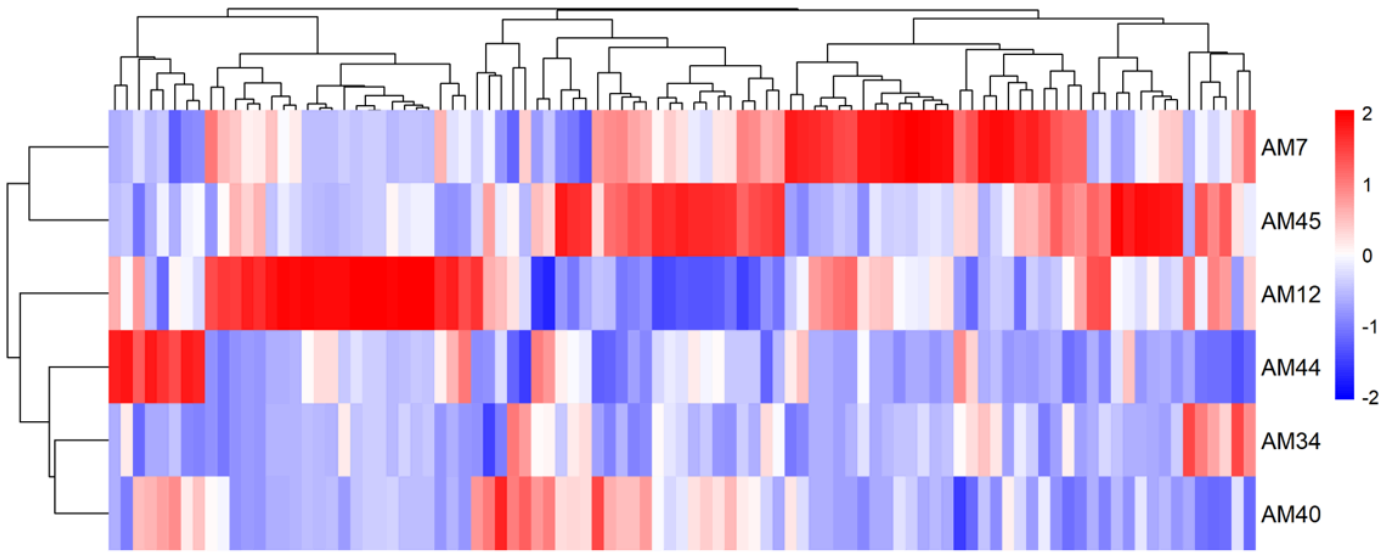

Figure 4

Heatmap scaled by columns to compare isolates across the same substrate. This column-scaled heatmap does not indicate which compounds are most actively metabolized by a given isolate, but rather highlights differences among substrate usage by the isolates. The list of substrates, from left

to right, can be found in Table S1.

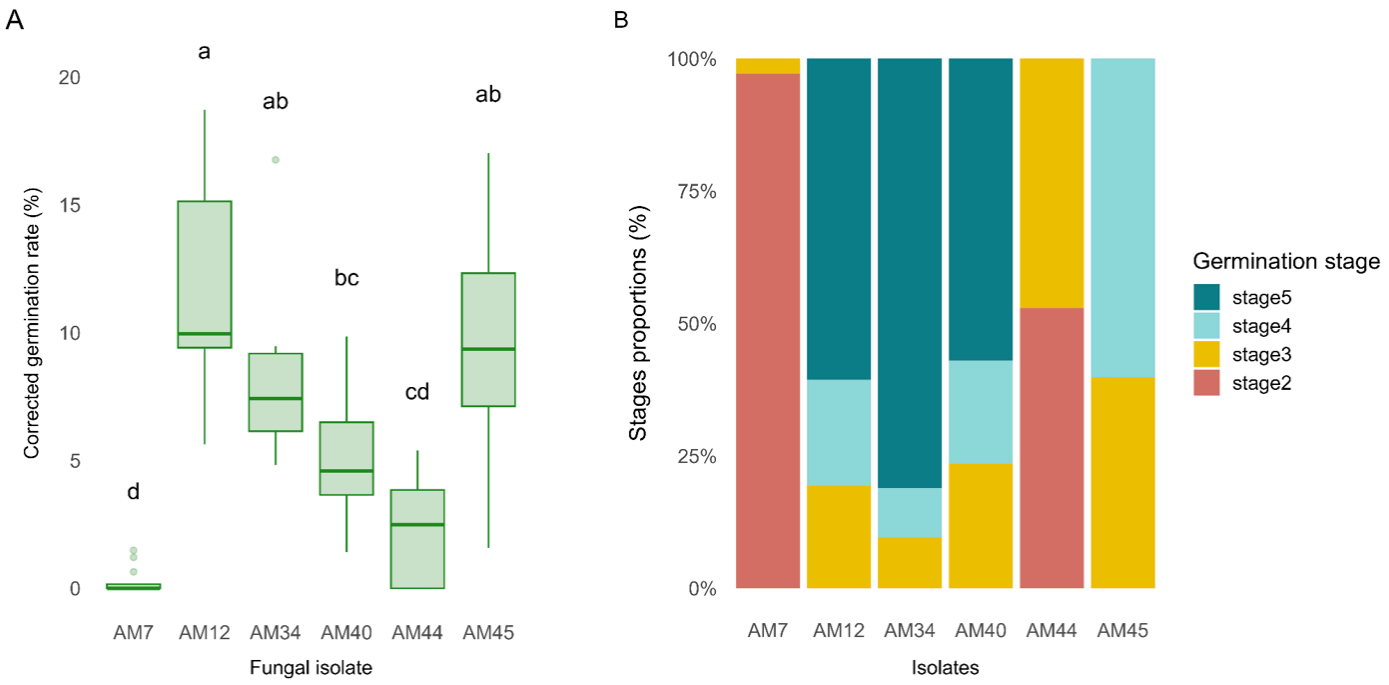

Figure 5

Germination tests with the six fungal isolates. A. Mean germination rate after 4 months calculated considering the seeds' viability of 72%. Boxplots show median values and significance groupings. B. Proportion of seeds reaching different germination stages (stage 2–5) for each isolate, referring to Swarts and Dixon (2017). Values are expressed as percentages.

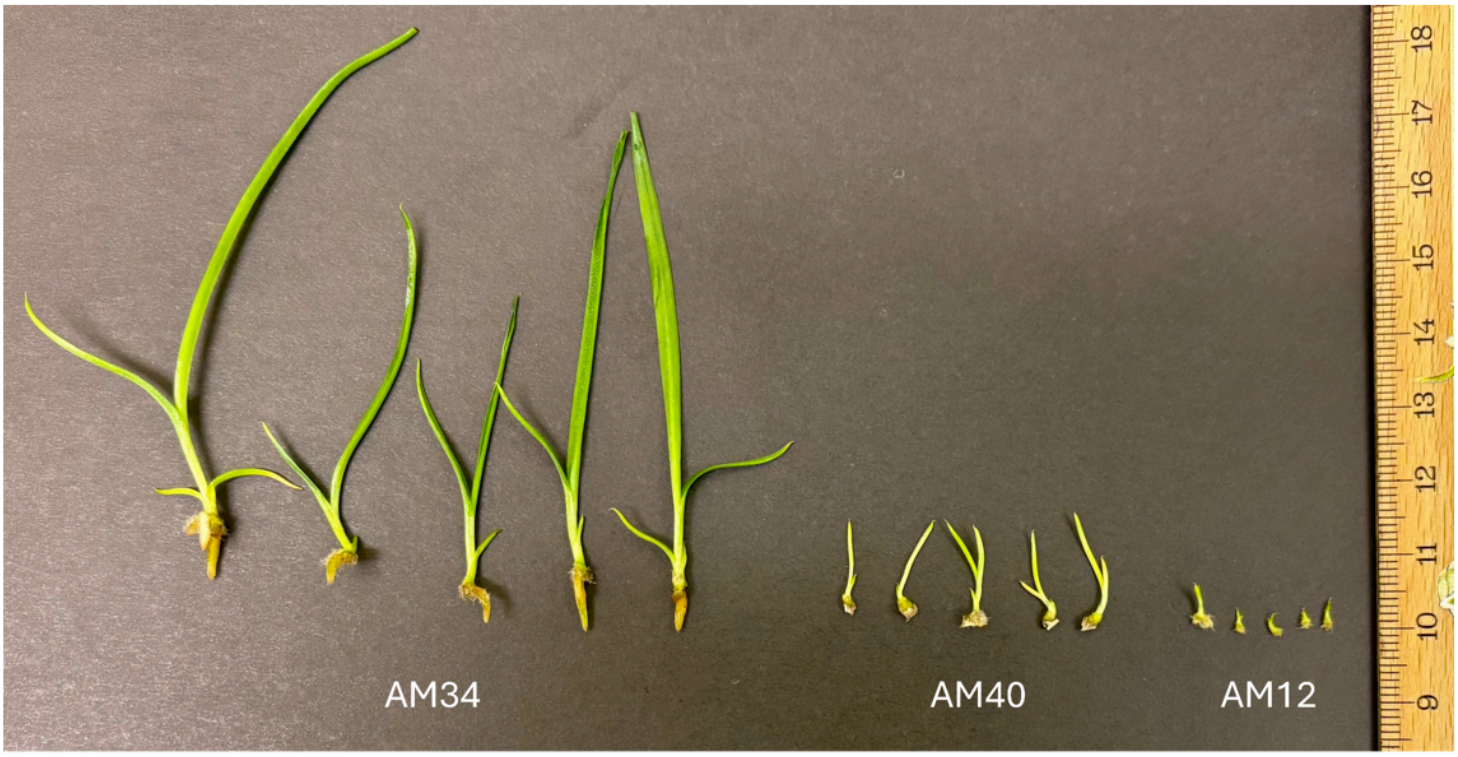

Figure 6

Development of mycorrhizal plantlets of *Serapias vomeracea*. Plants were photographed after six months of co-culture with the three fungal isolates that could induce leaflet formation. Centimetric scale bar.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.pdf